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Délivré par : *l'Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)*

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Marine Cambon

Heterogeneity within infections: the case of the vector-borne insect pathogen, *Xenorhabdus nematophila*

JURY

Claire Valiente-Moro	Maître de conférence	Rapporteuse
Dieter Ebert	Professeur d'université	Rapporteur
Franck Dedeine	Maître de conférence	Membre du Jury
Mathieu Sicard	Professeur d'université	Membre du Jury
Sophie Gaudriault	Chargée de Recherche	Directrice
Jean-Baptiste Ferdy	Professeur d'université	Directeur

École doctorale et spécialité :
SEVAB : Écologie, biodiversité et évolution

Unité de Recherche :
Évolution et Diversité Biologique, UMR UPS-CNRS-IRD, Toulouse
Diversité, Génomes et Interactions Microorganismes-Insectes, UMR UM-INRA, Montpellier

Directeur(s) de Thèse :
Jean-Baptiste Ferdy et *Sophie Gaudriault*

Rapporteurs :
Claire Valiente-Moro, *Dieter Ebert* et *Guillaume Mitta*

À Mutti

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Introduction

1 Infectious diseases and Pasteur-Koch heritage

In the late XIXth century, scientists discovered that microbes can be the cause of diseases. The establishment of this "GERM THEORY OF DISEASES"[1], opposed to the "SPONTANEOUS GENERATION THEORY" took an important turn in 1857 with the work of the French chemist Louis Pasteur. Pasteur discovered that, while yeasts were responsible for alcohol production during fermentation, wine and beer could be turned sour by another type microorganism (Pasteur 1857). Pasteur was convinced that these living microorganisms were the *cause* of liquid putrefaction, and not *arising* from putrefaction as taught by the "spontaneous generation theory". He also had the intuition that the same reasoning could apply to human, animal and plant diseases. Using a now famous experimental design (see Figure 1), he demonstrated in 1861 that the microbes responsible for liquid putrefaction were present in the air and that they could be killed by heating the liquid (Pasteur 1861). These findings stroke a fatal blow to the spontaneous generation

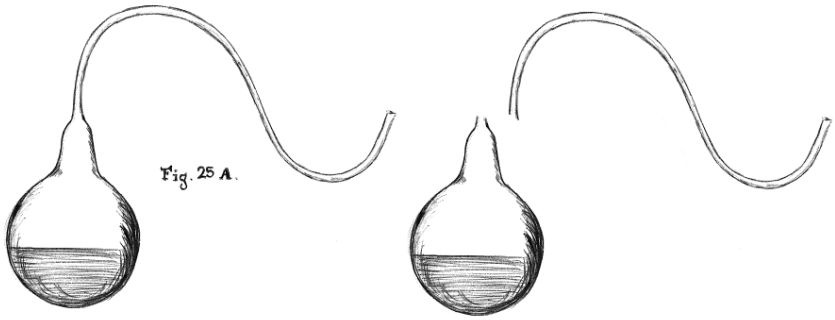

Figure 1 – **Copy of Pasteur's drawings of "swan-necked" flasks**. These flasks were made by Pasteur to refute the spontaneous generation theory. Pasteur boiled some broth into these flasks to sterilize it. The swan neck allowed air exchanges between the outside and the inside of the flask, but prevented the entrance of dust and airborne microorganisms which deposited by gravity in the lower section of the swan neck. Using this design, Pasteur showed that the broth remains intact until the swan neck is broken. Then, airborne microorganisms can enter the flask and colonize the broth. This experiment demonstrated that life cannot arise by itself inside the broth.

1. Words in capital letters in the text are defined at the end of this section.

theory, and participated to the initiation of reflections about the role of microorganisms in diseases. Later on, Pasteur conducted some work on silkworms, showing that pébrine disease, causing at that time disasters in the french silk industry was in fact due to a microorganism that could transmit from diseased to uninfected silkworms.

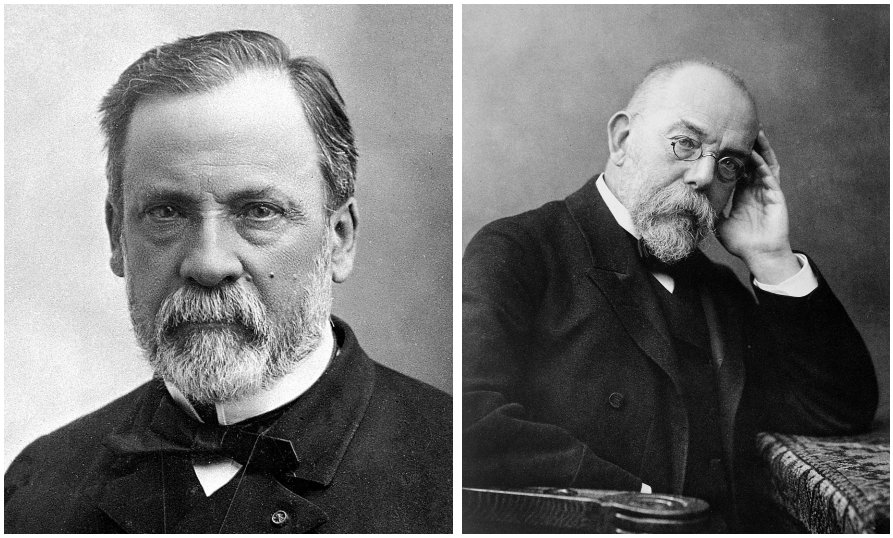

Figure 2 – **Louis Pasteur (left) and Robert Koch (right)** Photography by Paul Nadar and Wilhelm Fechner. Public domain, via Wikimedia Commons `https://commons.wikimedia.org/w/index.php?curid=422990` `https://commons.wikimedia.org/wiki/File:Robert_Koch.jpg`

In the meanwhile, the German physician Robert Koch worked on the cause of anthrax in cattle and sheep. In 1876, he isolated a bacteria from infected animals, and established for the first time the link between a specific microorganism and a specific disease (Koch 1876). Later on, Koch discovered the microbes that cause tuberculosis and cholera (Koch 1882). Now deeply convinced that some diseases were caused by microbes, he defined in 1882 four rules that he suggested to be strictly followed to demonstrate that a microorganism was the cause of a disease: (1) The organism must be present in every case of the disease. (2) The organism must be isolated from a host with the corresponding disease and grown in pure culture. (3) Samples of the organism removed from the pure culture must cause the corresponding disease when inoculated into a healthy, susceptible laboratory animal. (4) The organism must be isolated from the inoculated animal and identified as being identical to the original organisms isolated from the initial, diseased host (Lerner et al. 2006b; Lerner et al. 2006a).

The Pasteur and Koch heritage, together with the work of many other scientist from the XIXth century, led to a dogma, associating one microorganism to one disease. It allowed a huge jump in the understanding of infectious diseases, their epidemiology, as well as medical care and prevention.

2 Heterogeneity in infections and consequences from the pathogen point of view

The "germ theory of diseases" and Pasteur's and Koch's work basically described infections as interactions between one HOST and a single PATHOGEN. This classical model of single-species infection was criticised in the early years of the XXth century (Evans 1976). Scientists realised that infections could also be the consequence of several (sometimes many) different microorganisms and that some diseases could not be understood without taking into account complex multi-species interactions. I will use the term "heterogeneous infections" to designate these complex situations where a community of microbes all together contribute to the exploitation of a single host. Heterogeneous infections can arise in several distinct ecological situations discussed below, and in all cases, pathogens that are embarked in these communities may interact with many different microbes. Their pathogenicity, their transmission, and many of the traits that make them pathogens are most probably affected by these interactions. This is the phenomenon this thesis aims at surveying.

The understanding of heterogeneity in infections can be critical from a clinical point of view, as it can increase or moderate the detrimental effect of pathogens on their hosts. However, since the focus of my work is the pathogen itself rather than the host, **I will try to describe in the following lines the several forms of heterogeneity in infections and their consequences from the pathogen point of view**. I will mostly focus on what happens during the course of one infection. The term "pathogen populations" will thus refer to the populations of pathogens inside one host, during an infection, and not the population of pathogen at the ecosystem level.

2.1 Heterogeneity in the biotic environment of the pathogen, or inter-specific heterogeneity

Multiple infections: several pathogenic species or strains can infect a given host

The most intuitive way by which infections can be heterogeneous is when a host is infected by several pathogenic species at the same time, producing some inter-specific heterogeneity. A nice example of such multiple infections has been described in the field vole, *Microtus agrestis*. Telfer et al. (2010) showed that at least four different pathogens, including virus, protozoan and bacteria, can co-infect a host and interact with each other. For instance, they demonstrate that individuals previously infected by the bacterium *Anaplasma phagocytophilum* are more susceptible to infections by the protozoan *Babesia microti*. On the other hand, individuals that are previously infected by the bacterium

Bartonella spp. are less susceptible to *Babesia microti* infections. This mixture of facilitation and protection effects most probably result from direct or indirect (e.g. mediated by host immune defenses) interactions between pathogens inside the host body. The ability of a given pathogenic species to be transmitted cannot be understood here without taking into account the presence or absence of other pathogenic species inside the host.

Multiple infections may also involve different strains of a single pathogen species, rather than different species as in the previous example. In this case, the coexistence of closely related pathogens (strains or isolates), probably sharing the same ECOLOGICAL NICHE, implies potentially strong competitive interactions. This is documented, for instance, for the agent of the rodent malaria, *Plasmodium chabaudi*, where a single host can be infected by several parasite strains at the same time. These strains, with different levels of virulence, have been shown to compete with each other inside their host, as they all exploit the same resources: red blood cells (Bell et al. 2006).

Multiple infections can have several evolutionary consequences for pathogens. The most commonly assumed is that it should select for higher VIRULENCE in pathogen species or strains (Alizon et al. 2013). In the example of *Plasmodium chabaudi* cited above, strains that were able to more efficiently exploit red blood cells (i.e. with a higher virulence), reached higher densities when co-infecting a host with a less virulent strain (Bell et al. 2006). A POSITIVE SELECTIVE PRESSURE should thus act on these more virulent strains, while less virulent ones are counter selected as they are less efficient during multiple infections. The SELECTIVE PRESSURE that result from competitive interactions between pathogens are predicted to lead to the evolution of various strategies, such as niche divergence or production of interference molecules to prevent competition (see Mideo 2009, for a review).

Interactions with the host microbiota

Pathogens may not only encounter other pathogenic species during host infection but also a potentially diverse microbial community. Indeed, each single host individual shelters a huge amount of microorganisms, essentially non pathogenic microbes, called the MICROBIOTA. Pathogens that infect a host therefore do not colonize an empty environment, and their interactions with the host microbiota need to be taken into account to understand infectious processes and the evolution of pathogens (Belizário et al. 2015; Stecher et al. 2008).

The most complex and studied part of microbiota is that of the digestive tract of the hosts. Consequently, most studies of interactions between a pathogen and the microbiota of its host concern pathogens that pass through their host gut during their life cycle. For example, the human enteric pathogen *Salmonella enterica* serovar Typhimurium (*S.* Tm) needs to outcompete its host microbiota in order to invade the digestive tract. In the lab, mice with their native microbiota are resistant to colonization by *S.* Tm. Contrarily, mice

that have a reduced intestinal flora are susceptible to colonization. Using a METAGENOME analysis, Brugiroux et al. (2016) identified the functions fulfilled by the members of the mouse native microbiota that were essential for protection against *S.* Tm colonization. Doing so, they were able to reconstruct a minimal bacterial community that provides protection once established in germ-free mice. Here, the protective effect of the gut microbiota against pathogens arises from a wide range of functions fulfilled by multiple microorganisms.

In some cases however, specific members of the host gut microbiota can have a direct negative effect on pathogens through the production of deleterious molecules. It is the case for example for *Enterococcus mundtii*, a bacteria found in the gut of the lepidopteran *Spodoptera littoralis*. The SYMBIONT *E. mundtii* secretes an antimicrobial peptide into its host gut lumen. This peptide selectively kills opportunistic pathogens such as *E. faecalis*, but does not affect the resident gut flora (Shao et al. 2017). The specificity of this antimicrobial molecule toward a pathogen could suggests some strong selective pressure from pathogens on members of the host microbiota.

On the other hand, the host microbiota can have a beneficial impact on pathogens. For instance, the virulent potential of the entomopathogenic bacteria *B. thuringiensis* have been highly debated (see for example Raymond et al. 2010). *B. thuringiensis* produces a toxin that, when ingested by an insect, is responsible for intestinal wall perforation and subsequent insect death. The actual cause of host death have been thought to be the gut paralysis and associated feeding cessation that are observed in insects after toxin ingestion. However, a recent study showed that when insects do not control the gut microbiota (RNAi-mediated silencing of an immune gene), the lesions induced by *B. thuringiensis* allow the passage of bacteria through the intestinal barrier associated with a significant enhancement of host larvae mortality (Caccia et al. 2016). This septicemia enhanced by the host microbiota is thought to be in part the cause of insect death. Here, the host microbiota may have influenced the evolution of *B. thuringiensis* virulence factors that can be costly, and potentially useless in this case.

2.2 Heterogeneity in the pathogen population during the course of infection, or intra-specific heterogeneity

Within-host evolution

Heterogeneity can arise within the pathogen population itself, transforming a clonal infection into an assemblage of several pathogenic sub-populations (see Didelot et al. 2016, for a review). The simplest mechanism that cause this intra-specific heterogeneity is the accumulation of mutations while pathogens multiply in their host. These mutations, can allow ADAPTIVE EVOLUTION of pathogens during infection. An example of such host ADAPTATION have been shown in *Burkholderia dolosa*, which can opportunistically infect

humans suffering from lung fibrosis. Lieberman et al. (2011) showed that *B. dolosa* strains acquire within their host, and after the initial infection event, mutations in genes involved in resistance to antibiotics, which are usually administrated to patients suffering from lung fibrosis. They moreover found evidences of positive selection acting on genes that may undergo oxygen-dependent regulation, which could be crucial for the adaptation of free-living bacteria to the cystic fibrosis lung where oxygen availability is reduced. These findings suggest pathogens adaptation to a new ECOLOGICAL NICHE during infection. In some cases, within-host adaptation can be accelerated by a global increase of mutation rate, through the loss of functionality in DNA repair systems. For example, in some strains of *B. dolosa*, mutations into genes involved in DNA repair are associated to an excess of mutation, in the case of infection of a host with lung fibrosis (Lieberman et al. 2014).

Within host evolution can also be accelerated by HORIZONTAL GENE TRANSFER events, which allows exchanges of complete genes and functions between bacteria. For example, Stecher et al. (2012) showed that infection by *S. enterica* serovar Typhimurium promotes some bloom of *Escherichia coli* in the host gut, and that these blooms are associated with an increase of horizontal gene transfers via cell conjugation between *S.* Tm and *E. coli*. Here, infection can potentially promote the spread of virulence or antibiotic-resistance factors from pathogens to commensal bacteria. In any case, mutations and horizontal gene transfers initially bring some genetic diversity in the pathogen population, but the persistence of heterogeneity on the long run will depend on selection.

Phenotypic heterogeneity and immune evasion

While mutation events produce random variations at the scale of the whole genome, some mechanisms produce variation on specific genomic regions. One of them, quite common in bacteria, is phase variation. Phase variation is defined as a genetic or epigenetic mechanism resulting in a high rate of reversible phenotypic variation during the course of infection (van der Woude et al. 2004). One example of such phase variation is described in *Borrelia burgdorferi*, the agent of Lyme disease. The bacterium possesses two plasmids responsible of GENE CONVERSION events on genes encoding for ANTIGENIC MOTIFS. These conversion events, associated to duplicated genes transpositions produce a large number of distinct antigenic motifs, probably through modifications of surface proteins (Embers et al. 2004; Labandeira-Rey et al. 2003). This specific case of phase variation, called antigenic variation, allows the pathogen to escape its host immune system by producing a large diversity of antigenic motifs.

This escape of immune system through phase variation can also be mediated by epigenetic mechanisms. In *Salmonella enterica*, the modification of the O-antigen subunits of lipopolysaccharides present at the cell surface is involved in intestinal persistence in the mouse model. Indeed, modifying this O-antigen by the addition of some sugars allows to

escape the immune system. These additions of sugars are mediated by the *gtr* operon, which expression can be switched ON and OFF by DNA methylation (Garcia-Pastor et al. 2018).

Phenotypic heterogeneity, division of labour and bet-hedging

In many bacterial species, phase variation does not allow immune evasion, but rather produces phenotypic forms which lack key pathogenic functions (van der Woude et al. 2004; van der Woude 2011; van der Woude 2006). The fact that a sub-population of the pathogen which cannot accomplish the infection by their own are generated during the course of an infection can seem paradoxical but is well understood in the human pathogen *Salmonella enterica* serovar Typhimurium. This bacterium infects the digestive tract and breaches the gut wall of its host. It then colonizes lymphoid tissues, establishes into phagocytotic cells that carry them to the spleen and other systemic tissues. During its life cycle, *S.* Typhimurium exhibits heterogeneity in the expression of some genes (as described above). These differences in expression are stochastic (i.e. not triggered by environmental cues) and produce phenotypic heterogeneity.

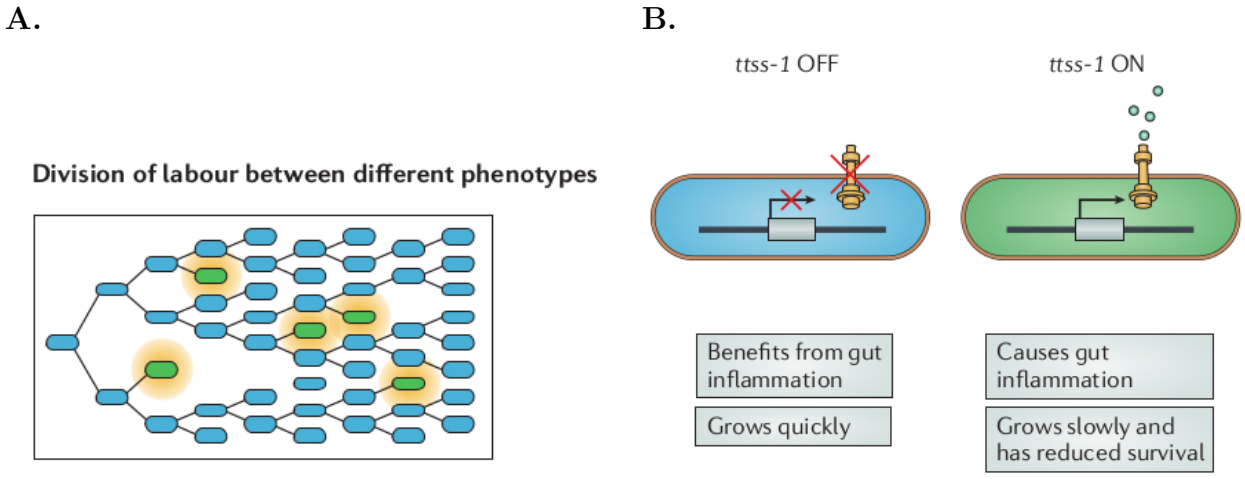

Figure 3 – **Phenotypic heterogeneity can lead to the division of labour in clonal groups.** This function of phenotypic heterogeneity manifests in infections with *Salmonella enterica* subsp. *enterica* serovar Typhimurium, used as an example here. **A.** Phenotypic heterogeneity can lead to interactions and the division of labour within clonal populations. For a genotype that expresses two different phenotypes (blue and green cells), individuals that express the green phenotype do not continue to grow but do produce a resource (orange) that promotes the growth of the blue phenotype. **B.** *S.* Typhimurium exhibits phenotypic heterogeneity in the expression of the virulence locus type three secretion system 1 (*ttss-1*), which encodes a multi-protein secretion apparatus. This leads to a division of labour between *ttss-1* OFF and *ttss-1* ON subpopulations. The *ttss-1* ON subpopulation invades host tissue and causes inflammation but suffers a reduction in growth and survival. The *ttss-1* OFF subpopulation benefits from the inflammation and proliferates. *Figure and legend adapted from from Ackermann et. al (2015)*

In the gut of the host, this heterogeneity consists mainly in the existence of two sub-populations: a slow-growing subpopulation of *S.* Typhimurium that expresses a viru-

lence gene (*ttss-1* ON) coexists with a fast-growing subpopulation that is phenotypically avirulent (*ttss-1* OFF). Cells with virulence functions induce inflammation mechanisms, which contributes to kill commensal bacteria in the digestive tract of the host (Kaiser et al. 2012). The avirulent form of *S.* Typhimurium, which do not pay the cost of expressing virulence factors, multiply without suffering from competitive interactions with the commensal bacteria, thanks to the virulence factors produced by the avirulent form (Figure 3a). Phenotypic heterogeneity allows a form DIVISION OF LABOURS where two subpopulations of the pathogen fulfill different functions (Figure 3b). The functional specialization of these subpopulations provides a competitive advantage to *S.* Typhimurium against other bacteria inside the host.

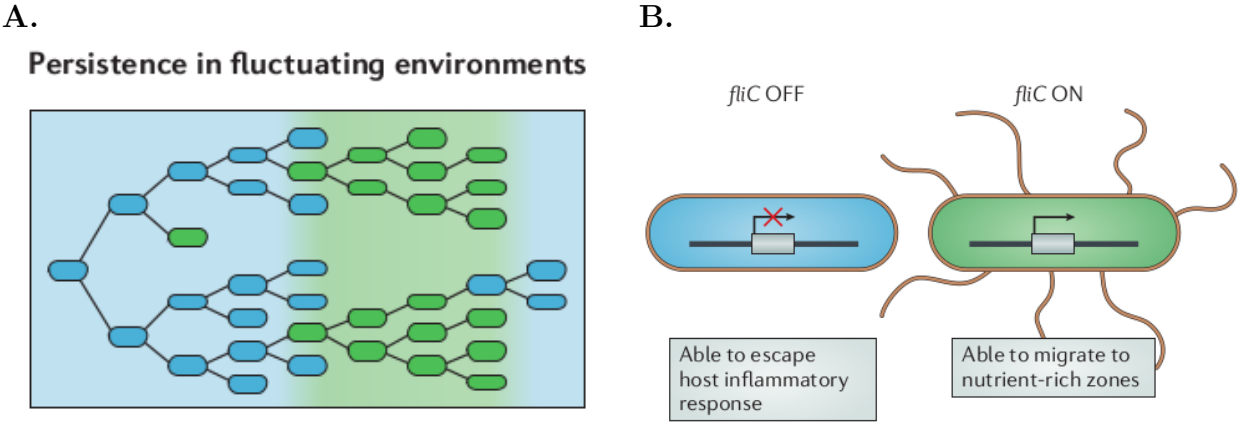

Figure 4 – **Phenotypic heterogeneity can promote persistence in fluctuating environments.** These function of phenotypic heterogeneity manifests in infections with *Salmonella enterica* subsp. *enterica* serovar Typhimurium, used as an example here. **A.** A genotype that expresses two different phenotypes (blue and green cells) can persist in an environment that fluctuates between two states (light blue and light green) in which only individuals that express the matching phenotype can survive. **B.** *S.* Typhimurium exhibits phenotypic heterogeneity in the expression of the flagellin gene fliC, and this allows this organism to persist in an environment that fluctuates between favouring flagellation (*fliC* ON) and selecting against it (*fliC* OFF). Here, *fliC* regulation allows to escape caspase-1 inflammatory response that is only effective in some of the host compartments *Figure and legend adapted from Ackermann et. al (2015)*

A second form of heterogeneity is described in *S.* Typhimurium after it has crossed the intestinal barrier of its host. It switches ON or OFF the expression of the flagellin gene *fliC* with a low probability and independently from environmental cues (Ackermann 2015; Stewart et al. 2011). This results in mixed population of flagellate and unflagellate cells. Flagellate cells are able to migrate toward nutrient sources but are suppressed by the host immune system in some compartments. Thus they only perform well in the tissues where these defenses are down. On the contrary, unflagellate cells can resist the host defenses but cannot migrate to reach nutrient sources (Figure 4B). Unflagellate cells are advantageous when the pathogen reach specific compartments. *S.* Typhimurium thus need to cope with a fluctuating and unpredictable environment as it migrates through different tissues of the host. The ON or OFF switching of the expression of the flagellin

gene *fliC* with a low probability and independently from environmental cues (Ackermann 2015; Stewart et al. 2011) can thus be interpreted as a BET-HEDGING strategy. This strategy consists in simultaneously producing different phenotypic forms, each adapted to a different type of environment. Doing so increases the chance that one of the phenotypic forms performs well in the current environment (Figure 4A).

2.3 The specific case of vector-borne diseases

In the above lines, we saw that contrarily to what was assumed by Pasteur and Koch, infections are often interactions between a host and a complex assemblage of organisms inside this host, potentially including non pathogenic microorganisms, several species and strains of pathogens, and several phenotypic forms of a given pathogenic strain. To make the picture even more complex, we must pay attention to the particular case of vector-borne diseases. These pathogens interact with another protagonist, the vector, which brings another source of heterogeneity in infections. Here again, I will not focus on differences that can exist between several vectors individuals, but rather discuss the implication of microbial community heterogeneity in the specific case of vector-borne diseases.

From the pathogen point of view, vectors can usually be seen as obligatory intermediate hosts. Consequently, all forms of heterogeneity described above can potentially apply in the vector environment. To my knowledge, few studies have investigated the role of phenotypic heterogeneity and within-vector evolution in vector-borne diseases. However, an increasing number of studies concern the role of vector microbiota in the transmission of human diseases. It has been shown for instance, that mosquito microbiota influences vector competence to the transmission of the human parasite *Plasmodium falciparum*. In particular, members of the mosquito microbiota could reduce the vector colonisation by *P. falciparum* through competition for resources, immune priming, and production of secondary metabolites (Cirimotich et al. 2011; Weiss et al. 2011; Dennison et al. 2014). On the other hand, some bacterial taxa from the vector microbiota could have a positive effect on *P. falciparum* colonization, as showed for Enterobacteriaceae in Boissière et al. (2012)

The same kind of observations hold for the agent of Lyme disease, *Borrelia burgdorferi* and its tick vector, *Ixodes scapularis*. Narasimhan et al. (2014) showed that the alteration of the tick gut microbiota altered in return its intestinal peritrophic matrix. This peritrophic matrix being essential for *B. burgdorferi* colonisation, dysbiosed ticks are less colonized by the pathogen. Here again, the vector microbiota plays a crucial role for pathogen transmission.

In the two examples cited above, members of the vector microbiota can probably be transmitted together with the pathogen. They can potentially play a role in the infectious

process, and interact with the pathogen. Although studies on the role of vector microbiota on pathogen transmission are increasingly conducted, very few have focused on role of vector microbiota in the infectious process within the host.

3 *Xenorhabdus-Steinernema* complexes: combination of multiple heterogeneity levels

3.1 Entomopathogenic nematodes-bacteria pairs

Xenorhabdus and *Photorhabdus*, two closely related bacterial genera, belonging to the Enterobacteriaceae family (γ-Proteobacteria), are mutualistically associated with nematodes from *Heterorhabditis* and *Steinernema* genera, respectively. These nematodes, which have a very similar life cycle (see section 3.2), are found in soil where they infect a wide variety of insect larvae (Peters 1996). Due to their large distribution around the world and their potential interest in pest control, entomopathogenic nematodes, and their bacterial symbiont have been the object of many studies.

This work is focused on the *Xenorhabdus* bacterial genus, which includes several species, associated with several *Steinernema* nematode species. A tight co-evolution between *Xenorhabdus* and *Steinernema* species is supported by the fact that the *Xenorhabdus-Steinernema* symbiosis is very specific: nematodes of a given species only "allow" one species of *Xenorhabdus* to colonize their gut. Some close relative species from the *Xenorhabdus* genus can be artificially re-associated to non-native *Steinernema* species, but the benefit (see section 3.2 for details) they bring to the nematode is usually much lower than that brought by the native symbiont, and they cannot be transmitted by the nematode descendants (Sicard et al. 2004b; Chapuis et al. 2009). This specificity is permitted by a complex molecular dialogue between the two parts of the symbiosis which as been well described in *X. nematophila-S.carpocapsae* (Heungens et al. 2002; Goodrich-Blair 2007; Chaston et al. 2013). Surprisingly, this specificity of association does not seem to result from co-speciation events, as suggested by the low relatedness of the two taxonomic structures of *Xenorhabdus* genera and their host nematodes (Figure 5 Boemare (2002) and Lee et al. (2010)).

3.2 *Xenorhabdus-Steinernema* life cycle

Only differentiated L3 larval stages of nematodes disperse in the soil. These infective juveniles (IJs) are protected by both L3 and L2 cuticles, the later obstructing their mouth and anus which prevent them from feeding, but allow them to persist inside the soil (Sicard et al. 2004a). These IJs are colonized by an almost clonal population of *Xenorhabdus* in an intestinal receptacle (Martens et al. 2003). The IJs disperse in soil until they find a

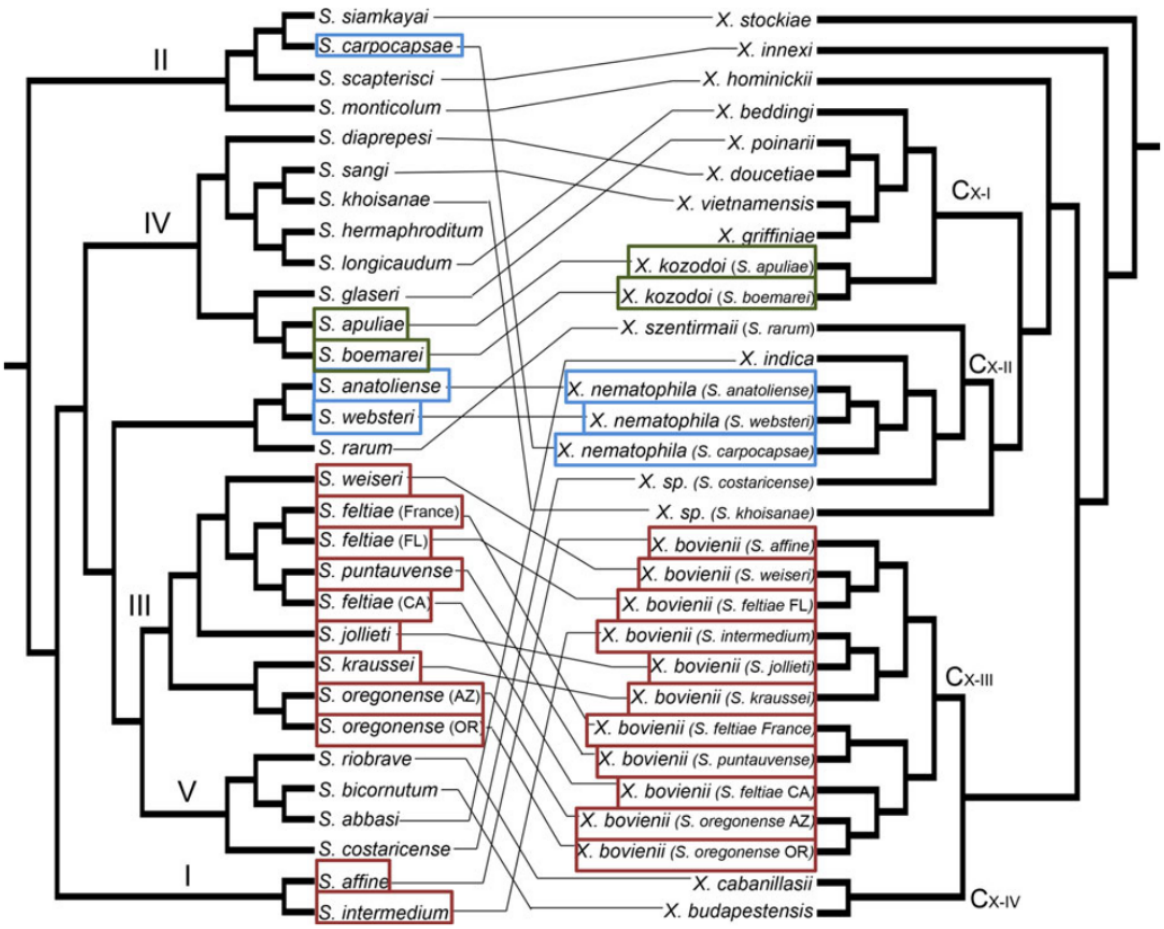

Figure 5 – Multi-locus cophylogeny of nematode species from the *Steinernema* genus, and their symbiont from the *Xenorhabdus* genus. Coloured squares represent different strains of the same *Xenorhabdus* species. Each *Steinernema* species are linked to their native symbiont strain. Nematode phylogeny is based on one nuclear, 28S rRNA, and two mitochondrial genes, *COI* and 12S rRNA. Bacteria phylogeny is based on the 16S ribosomal gene, and two housekeeping genes, *serC* and *recA*. *Figure from Lee et al. (2010)*

larval insect host to invade (Figure 6.1). They usually penetrate the insect larvae through natural openings like mouth and anus. Within the larvae of the host insect (*e.g. Galleria mellonella* or *Spodoptera* sp. in lab conditions), nematodes perforate the intestinal wall and releases their *Xenorhabdus* symbionts into the insect hemolymph (Figure 6.2) (Sicard et al. 2004a; Snyder et al. 2007). The bacteria grow to high densities into the extracellular matrix of the host tissues (Sicard et al. 2004a), reduce the host immune response through haemolysin production, which targets the host hemocytes (Vigneux et al. 2007), inhibition of antimicrobial peptides production (Park et al. 2007) and phenol-oxidase activity (Crawford et al. 2012). The insect host dies of septicemia in approximately 24h (Dowds et al. 2002; Richards et al. 2009). *Xenorhabdus* then produces enzymes which degrade insect tissues to provide nutrients for the nematodes (Caldas et al. 2002; Chen et al. 1996). Nematodes perform several cycles of reproduction within the insect cadaver (Figure 6.3).

When nematode number becomes high and nutrients are limiting, *Steinernema* juveniles re-associate with *Xenorhabdus* and differentiate into non-feeding IJs. The IJs finally disperse into the soil in the search of a new host (Figure 6.1). For simplicity purposes, IJs of *Steinernema* will be designate as *Xenorhabdus* vector, although they also have a role to play in killing the host.

3.3 *Xenorhabdus nematophila*, a model species

Among these bacteria associated with entomopathogenic nematodes, *Xenorhabdus nematophila* have been extensively studied because of its particularly high virulence toward many model insects (McMullen et al. 2017; Ogier et al. 2014; Kim et al. 2017). Moreover, *Steinernema carpocapsae*, its nematode vector, can be experimentally deprived of its symbiont (Sicard et al. 2003). These aposymbiotic nematodes are of great interest to perform re-associations with other strains or species of symbionts, and investigate evolutionary questions. For example, the interaction between *X. nematophila* and its vector *S. carpocapsae* can be considered as mutualistic because, first, the bacteria cannot survive more than a few days in the soil unless they are carried by a nematode (Morgan et al. 1997) and, second, symbiotic *S. carpocapsae* (i.e colonized by *X. nematophila*) have a much higher reproductive rate than aposymbiotic nematodes (Sicard et al. 2003). This later observation might be attributed to (1) a more effective inhibition of the insect immune system, (2) a better nutrition of the nematode in the insect when associated with *X. nematophila* (Richards et al. 2009) and (3) a protection against 'enemies', by producing antimicrobial molecules that could eliminate microbial competitors encountered in soils or in insect cadavers (Singh et al. 2015), or dissuading insects from feeding on its insect-host cadaver (Zhou et al. 2002). On the other hand, some studies have shown that the survival rate of the IJs decreases when they are associated to *X. nematophila* (Emelianoff et al. 2007; Emelianoff et al. 2008a). It suggests that nematodes experience a survival-reproduction trade-off which is induced by the symbiosis, and which probably has shaped the co-evolution between the two partners (Chapuis et al. 2012).

The fact that we can produce aposymbiotic IJs of *S. carpocapsae*, that we can reproduce the whole life cycle of *S. carpocapsae-X. nematophila* pairs in lab conditions, and that the whole genome of *X. nematophila* F1 strain has been sequenced (Lanois et al. 2013) makes *X. nematophila* a very convenient model species. Moreover, several aspects of its biology that I will describe further make it very interesting to study heterogeneity in infections.

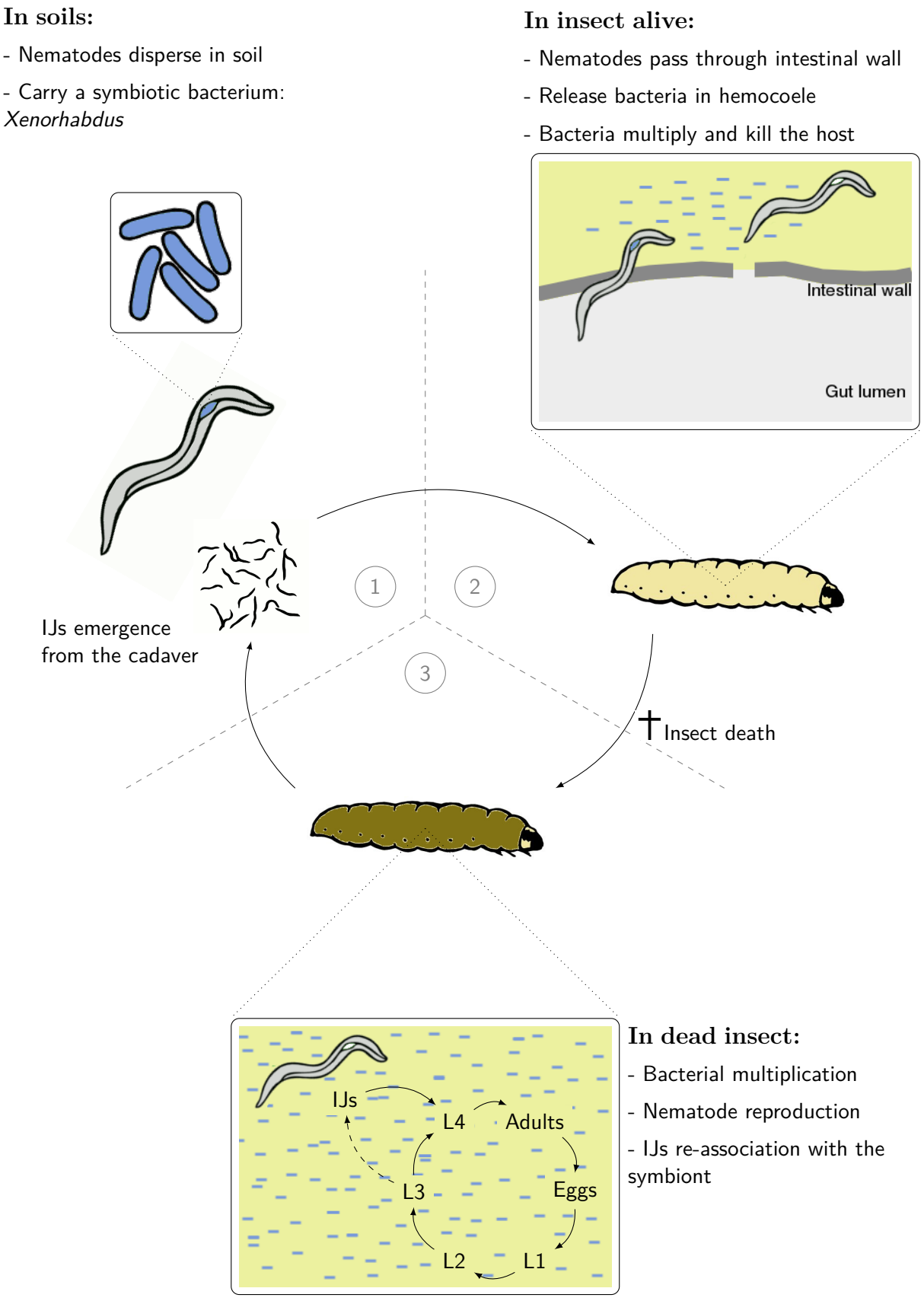

Figure 6 – Life cycle of the *Xenorhabdus* bacterium carried by its nematode vector *Steinernema* and infecting the insect host *Galleria mellonella* larvae usually used in laboratory experiment. IJs stands for infective juveniles, which are specific L3 stages that did not lose their L2 cuticule.

3.4 Heterogeneity in *Xenorhabdus-Steinernema* infections

Intra-specific heterogeneity

X. nematophila isolated from the symbiotic nematodes found in soil has the particularity to adsorb bromothymol blue. Therefore, it forms blue colonies when it grows on agar containing this dye (Figure 7). When these blue colony-forming cells, referred as group 1, are grown in *in vitro* culture, a second form of *X. nematophila* called group 2 may appear after several days of incubation (Figure 7). This group 2 form produces red colonies on the same culture medium (Akhurst 1980). Group 2 cells also present many phenotypic differences with group 1 cells: they have a reduced antimicrobial (Akhurst 1980), proteolytic and lipolytic activity (Thaler et al. 1998), and a reduced production of flagellar filaments (Volgyi et al. 1998; Givaudan et al. 1996; Givaudan et al. 1995). Although group 2 are usually obtained in *in vitro* cultures, Akhurst (1980) reported that these phenotypic variants of *X. nematophila* could also arise during insect infection. These phenotypic variants have been known and studied for the past four decades, yet the molecular mechanisms responsible for the production of heterogeneity, as well as their implication during host infection and re-association with the vector, are not fully understood. I will try in the first chapter of this thesis to bring some answers to these questions.

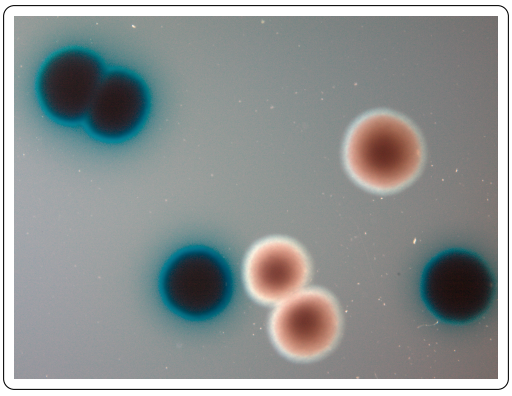

Figure 7 – Group 1 (blue) and group 2 (red) colonies of *Xenorhabdus nematophila* obtained by plating a stationary phase culture on solid medium containing bromothymol blue

Another type of intra-specific heterogeneity that will not be further developed in this thesis but deserves to be mentioned, is the fact that several strains of *X. nematophila* can infect a host at the same time. Indeed, multiple nematodes should enter the insect, as *Steinernema* reproduction is sexual. To cope with this intra-specific competition within hosts, *Xenorhabdus* species produce some toxins that can kill unrelated strains of the same species (Hawlena et al. 2010a). In several species of *Xenorhabdus*, including *X. nematophila*, these spiteful interactions between unrelated strains due to bacteriocin production benefit kin, i.e. closely related cells, but reduce the combined virulence of competing strains (Vigneux et al. 2008; Bashey et al. 2012). Moreover, these anti-competitor

abilities have been shown to trade-off against nematode reproductive success (Bertoloni Meli et al. 2018). This trade-off, together with the fact that in nature, non-inhibiting isolates can be found in high frequency, suggest that there may be two different strategies for pathogen success: one competitive strategy, which would be favored in competition with sensitive strains, and one reproductive strategy, favored in a non-competitive context (Bertoloni Meli et al. 2018). This context-dependant fitness supports the idea that intra-specific competition, associated with an heterogeneous environment, may maintain diversity in the pathogen population [2], and highlights the role of intra-specific competition in mutualism evolution.

Inter-specific heterogeneity

Similarly, several species of *Xenorhabdus* can infect one host at the same time. Bashey et al. (2013) showed that these inter-specific interaction of *Xenorhabdus* can favor higher virulence, or stains that are able to produce bacteriocin as described above. However, *Xenorhabdus* is also able to produce a large number of antibiotics that affect bacterial competitors from other genus (Singh et al. 2008; Singh et al. 2015; Park et al. 2009). This suggests that interactions with other bacteria may be a strong seclective pressure for *Xenorhabdus*. Indeed, the bacteria have to multiply and stay for around ten days inside an insect cadaver, were multiple competitors can develop. These competitors can potentially originate from the soil, but also from the insect microbiota, and from the nematode microbiota. It have been commonly assumed that producing these antibiotics was a way for *Xenorhabdus* to dominate the bacterial community inside insect cadavers. In the second chapter of this thesis, I focused on the bacterial communities that can be found in insect cadavers.

2. Here I mean the population of pathogens in a given environment, opposed to the term "pathogen population" within one host

4 Objectives of the present thesis

Chapter 1: A role of phenotypic heterogeneity in *Xenorhabdus nematophila* infections? In the first chapter of this manuscript, I investigated the potential role of the generation of phenotypic variants of *X. nematophila* during infection. The first part brings some answers about the genetic mechanisms that generate these phenotypic variants. I then investigated the potential adaptive value of these phenotypic variants during *X. nematophila* life cycle.

Chapter 2: Microbial communities during *X. nematophila* infections In the second chapter, I investigated the composition of microbial communities during *X. nematophila* infections. First, we developed an experimental procedure that allows to modify the gut microbiota of lab-reared insect in order to mimic soil-dwelling insects. We then used these insects colonized by a relevant set of microorganisms to describe the microbial community composition inside insect cadavers at the time-point of the re-association between *X. nematophila* and its vector.

Glossary

Adaptation: A trait that has been fixed by adaptive evolution, i.e. because it conferred greater fitness to its carrier.

Adaptive evolution: Changes of a trait frequency in a population due to natural selection (i.e. different from evolution due to genetic drift). We will use the phrase "adaptive evolution" as an equivalent to Darwinian evolution.

Antigenic motifs: Any external structure of a cell which can be recognised by a host immune system.

Bet-hedging: Evolutionary strategy where different phenotypes are produced independently of environmental cues, which increases the chance that at least one phenotype will perform well in one state of a fluctuating environment.

Division of labours: Evolutionary strategy where individuals within a group specialize in certain tasks to benefit the whole group.

Ecological niche: The set of biotic and abiotic conditions that allows the persistence of a given species.

Fitness: The average number of gene copies transmitted to the next generation.

Gene conversion: Homogenisation of homologous regions from two DNA strands that initially contained different alleles. As a result, the recipient strand carry the same allele as the donor strand.

Germ theory of diseases: A XIXth century theory claiming that microbes can be the *cause* of some diseases, and not their *consequence*, as taught by the spontaneous generation theory.

Horizontal gene transfer: Exchange of genomic material between two bacterial cells from different strains/species.

Host: Here refers to any multi-cellular organism that can be colonized by microorganisms.

Metagenome: The set of all the genomes of the cells from a bacterial population.

Microbiota: All microorganisms associated to a host at one time point.

Pathogen: Here refers to microorganisms (virus, bacteria, protozoans) which have a detrimental effect on a host.

Phase variation: A stochastic and reversible change of phenotype in bacteria, caused by a genetic or epigenetic mechanism.

Selective pressure: Any cause for an organism with a particular trait to have a fitness advantage (positive selective pressure) or disadvantage (negative selective pressure).

Spontaneous generation theory: Theory according to which living beings could arise from inanimate matter.

Symbiont: Microorganism living in close association (positive, negative or neutral) with a macroorganism.

Trade-off: Constrains (e.g. physical or physiological) that make it impossible to invest resources in two life-history traits at the same time. Trade-offs usually result in a negative relation between those traits. From an evolutionary perspective, they can lead to a compromise that consists in intermediate values of traits, or conversely to specialization, with the possibility that specialists coexist in populations.

Virulence: The reduction of a host fitness induced by a pathogen.

1 A role of phenotypic heterogeneity in *Xenorhabdus nematophila* infections?

Cells of *Xenorhabdus nematophila* that are isolated from nematodes usually form blue colonies when plated on agar containing bromothymol blue. After a prolonged *in vitro* culture, or injection in insects of these blue colony-forming cells, some cells forming red colonies appear. Compared to blue cells (primary variants) red cells (secondary variants) lack motility, and have a reduced production of enzymes and antimicrobial compound (Boemare et al. 1988; Givaudan et al. 1995; Boemare et al. 1997; Volgyi et al. 1998; Forst et al. 2002). I will call group 1 and group 2 the cohort of variants whose phenotypes correspond to that of primary and secondary variants historically described.

Several terms have been used so far to name this production of different phenotypes: phase variation, phenotypic variation, phenotypic switching, phenotypic heterogeneity. Most often, different scientific communities use different words. For the present work, which is at the intersection of microbiology and evolutionary biology, I chose to use the term PHENOTYPIC SWITCHING to designate the production of different contrasted phenotypes, called PHENOTYPIC VARIANTS, which results in PHENOTYPIC HETEROGENEITY in a bacterial population, without any prior on the mechanisms involved.

In fact, in *Xenorhabdus*, the mechanisms responsible for the production of phenotypic variants have not been fully understood, although some clues about the implication of the *lrp* gene have been showed. Lrp (leucine-responsive regulatory protein) is a global regulator of expression in *X. nematophila*, and *lrp* mutants exhibit a phenotype similar to that of group 2 phenotypic variants (Cowles et al. 2007). Moreover, a constant expression of *lrp* suppresses phenotypic switching (Hussa et al. 2015). Although *lrp* is pointed out in the production of phenotypic variants, the exact molecular mechanism remains to be elucidated.

Apart from the molecular mechanism that allows the emergence of these variants, their potential role in *X. nematophila* life cycle also have to be clarified. It is probably not a way for *X. nematophila* to escape the host immune system. Most of the genes whose expression differs between group 1 and 2 cells are indeed expressed long after host

death (Jubelin et al. 2013). Moreover, both group 1 and group 2 cells are able to kill their host when injected into the insect (Volgyi et al. 1998). The reduced activity of some genes in group 2 variants have been shown to be detrimental to the nematode, however group 1 and group 2 are both able to re-associate with nematodes and group 2 have been shown to be preferentially carried by nematodes when a mixture of the two groups was injected into an insect (Sicard et al. 2005; Richards et al. 2008). All these findings are somehow contradictory with the fact that nematodes freshly sampled from the wild have never been reported to carry group 2 variants. This paradox could come from the fact that some conditions favor the multiplication and transmission of group 1 variants, while other favor that of group 2 variants but are rarely encountered in nature. In any case, the interactions between group 2 variants and nematodes still need to be examined in details to better understand whether or not this phenotypic switching could be adaptive in the entomopathogenic symbiosis.

The aim of this work was to bring some answers to the following questions: **Which molecular mechanisms are responsible for the production of phenotypic heterogeneity in *X. nematophila* populations? Are these mechanisms adaptive?** We constituted a collection of 34 variants which we then characterized, both phenotypically and genetically. This was made collectively with teams from EDB lab in Toulouse, and DGIMI lab in Montpellier. My contribution to this work was to measure the virulence of the 34 variant when directly injected into insects, and test their adaptive nature *in vivo*.

Prolonged culture and long lasting infections select for poorly transmitted bacterial variants

Marine Cambon*†[1], Nathalie Parthuisot*[1], Sylvie Pagès[2], Anne Lanois[2], Alain Givaudan[2] and Jean-Baptiste Ferdy[1]

[1]Évolution et Diversité Biologique - UMR 5174 CNRS-UPS Université Paul Sabatier - F-31062 Toulouse cedex 9

[2]DGIMI - UMR1333 INRA -Université Montpellier - F-34095 Montpellier cedex 5

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*The first two authors equally contributed to this work

†cambonmarine@gmail.com

Abstract

Bacterial infections are often composed of cells with distinct phenotypes, that can be produced by genetic or epigenetic mechanisms. Heterogeneity in the course of infection has proved to be important in many pathogens, because it can alter both pathogenicity and transmission. We studied a large cohort of phenotypic variants of the insect pathogen *Xenorhabdus nematophila*. This bacterium kills insects and multiplies in the cadaver before being transmitted by the soil nematode vector *Steinernema carpocapsae*. We found that, based on several phenotypes, variants are clustered in three groups, one of which corresponding to mutations in the gene encoding the major regulator Lrp. These Lrp defective mutants survive better and reach high loads during prolonged stationary phase, which probably explains why they increase in frequency during infections. The third group of variants is less frequent, bears no mutation in *lrp* but is also advantaged in late stationary phase. Both group 2 and group 3 variants thus have a Growth Advantage in Stationary Phase (GASP phenotype), but we found evidences that this advantage trades off against transmission by nematodes: the highest load a variant reaches in insects, the least it is transmitted by nematode vectors.

Introduction

Bacterial infections, even started from a single pathogenic strain, often end up being composed of cells with different phenotypes. In cases where groups of cells with distinct phenotype interact to better exploit their host (Diard et al. 2013), the molecular mechanisms that produce this diversity can be considered as adaptations, which ultimately increase pathogens transmission. To demonstrate this theory, it is necessary to understand both what these mechanisms are, and how they impact the success of the infection.

Mutation is probably the most obvious mechanism that produces diversity during infection. Its importance is increasingly admitted (Didelot et al. 2016), notably because cases are now accumulating where it fuels the evolution of pathogens inside the host, in particular in human diseases (e.g. Markussen et al. 2014; Young et al. 2017). While mutation generally occurs throughout the whole genome, other mechanisms exist that impact a restricted set of genes. These can be epigenetic alterations, where clonal populations of bacteria modify their phenotype by changing their regulatory state (van der Woude 2006; van der Woude 2011). These can also be genetic alterations, as in phase variation where a specific high rate mutation mechanism produces reversible changes in one or a few genes (van der Woude et al. 2004; Bayliss 2009). Phase variation is described as the basic mechanism that makes antigenic variation a successful instrument for some pathogens to escape their host immune system (e.g. Zhang et al. 1997).

The insect bacterial pathogen *Xenorhabdus* is a promising model to study the

adaptive nature of mechanisms that control phenotypic variation inside an infection. This pathogen indeed kills insects and proliferates in their cadaver for one or two weeks, before it is transmitted by the nematode vector *Steinernema* (Goodrich-Blair 2007). During this long period, *Xenorhabdus* maintains high densities inside the insect and, therefore, potentially accumulates phenotypic variation. As the complete life cycle of *Xenorhabdus* can be experimentally reproduced (Chapuis et al. 2012), it is possible to quantify how this variation impacts each of its different stages.

Xenorhabdus isolated from the wild typically are in a form described as primary, but in culture media they convert to another, secondary, form when reaching long-term stationary-phase (Boemare et al. 1988). In its seminal paper, Akhurst (1980) showed that secondary forms of *Xenorhabdus* also appear during infection, and that this occurs at a rate that greatly varies among *Xenorhabdus* strains. Although the phenotypic differences between the two forms can also vary depending on strain and species, the primary but not secondary form cells are able to bind bromothymol blue dye, are motile, agglutinate red blood cells and produce fimbriae, haemolysins, proteases, antimicrobials and crystalline inclusion bodies (Boemare et al. 1988; Givaudan et al. 1995; Boemare et al. 1997; Volgyi et al. 1998; Forst et al. 2002). The alternation between primary and secondary forms has so far been interpreted as a case of phase variation (Boemare et al. 1988). As the phenotype of secondary forms matches that of *lrp* defective mutants (Cowles et al. 2007; Hussa et al. 2015; Cao et al. 2017; Casanova-Torres et al. 2017; Engel et al. 2017), it has also been proposed that the Lrp master regulator could control the production of secondary variants in *X. nematophila* (Cowles et al. 2007). It is not demonstrated, though, that *lrp* is systematically involved when this phenomenon occurs during *Xenorhabdus* infections. Interestingly, genes with differential expression between primary and secondary forms have also been shown to play a role in the interaction between *X. nematophila*, its nematode vector and its insect target (Richards et al. 2009), demonstrating that the production of secondary forms should impact *X. nematophila* interactions with their invertebrate hosts. However, despite reduced production of virulence factors in secondary forms, all forms of *X. nematophila* are capable to kill in insects (Volgyi et al. 1998). In addition, Sicard et al. (2004a) showed that both primary and secondary forms could be transmitted by the nematode *S. carpocapsae*. The goal of this paper is to better understand first, the molecular mechanisms that are responsible for the production of phenotypic variants, and second, the impact of these variants on the transmission by the vector. To do so, we investigated a large collection of *X. nematophila* isolates with various phenotypic forms. We found that secondary forms are not phase variants but rather plain *lrp* mutants, and that at least a third phenotypic form exists in *X. nematophila* which is not a *lrp* mutant. All these variants have a Growth Advantage in Stationary Phase (GASP, Finkel 2006) which probably explains why they reach

higher loads than primary forms during late infection. We then quantified how these variants are transmitted by the nematode vector *S. carpocapsae* and found that isolates that reach the highest densities in insects are the least transmitted by nematodes. *X. nematophila* therefore seem to experience a trade-off between traits that are favored during late infection and traits that increase transmission.

Materials and Methods

Obtention of X. nematophila isolates

Xenorhabdus nematophila isolates were obtained from static cultures of the GFP-labelled strain F1D3 (Sicard et al. 2004a). Samples of ten independent LB cultures of F1D3 were streaked onto NBTA plates (Akhurst 1980) after 3, 7 and 13 days of incubation at 28 °C. Primary forms form blue colonies on NBTA while secondary variants form red colonies (Boemare et al. 1988; Givaudan et al. 1995; Boemare et al. 1997). Each time a red colony was observed, a blue colony from the same Petri dish was also sampled. Overall, we obtained 34 distinct isolates (see Supp. Mat. 1) which we stored in 20 % glycerol at -80 °C.

lrp sequencing

X. nematophila colonies were lysed in sterile miliQ water after several freezing-thawing cycles. *lrp* gene was amplified using the high-performance GoTaq G2 DNA Polymerase (Promega, France) with primers lrp-L (5'-CATATTGCGGATTTAGGG-ATTG-3') and lrp-R (5'-GGGACTGCATAGGCAAGAATAC-3'). PCR products were sequenced by GenoScreen, France and mutations were determined by comparing aligned *lrp* sequences to that of the *X. nematophila* F1 reference genome (Lanois et al. 2013).

Measuring colony phenotypes

For each isolate, we measured four phenotypic traits that differ among *Xenorhabdus* variants. Swimming motility was measured as the diameter of a halo formed by motile bacteria on 0.35 % agar culture medium (Givaudan et al. 1995; Boemare et al. 1997). Antibiotic activity was quantified by measuring the diameter of inhibition halos, using *Micrococcus luteus* as a target strain (Givaudan et al. 2000). Extracellular lipolytic activity was assessed by the presence of precipitated material surrounding the colony cultured on Tween 20 agar (Givaudan et al. 2000). Haemolytic activity was quantified by the presence of a clearing surrounding bacteria grown on standard sheep blood agar plates (Vigneux et al. 2007).

Measuring cell size

Cell size was first estimated by flow cytometry analysis on three replicates *in vitro* experiments. In each experiment, exponential phase cultures of each isolate were fixed with a 0.2 % solution (v/v) of formaldehyde and analyzed with FACS-Calibur flow cytometer (Becton Dickinson), equipped with an argon air-cooled laser providing 15 mW at 488 nm and the standard filter set-up. Bacterial cells were discriminated from particles by applying a polygonal gate to green (530±15 nm) and red (585±21 nm) log-transformed fluorescence measurements and a rectangular gate on log-transformed side scatter (SSC) and forward scatter (FSC), using tools provided in R Bioconductor (Huber et al. 2015). We used log-transformed FSC as a proxy for cell size.

In other experiments, when cytometry was not applicable, we sampled colonies, put them on cover slides and took pictures with a Olympus BX51 microscope with 400× magnification. Images were analyzed using ImageJ (Schneider et al. 2012), the size of a cell being approximated by the maximum Feret diameter of the cell contour. We measured a minimum of 10 cells per sample and used the average Feret diameter (in μL) as a cell size estimate.

Measuring bacterial density and survival

In vitro bacterial density during stationary phase was measured on agitated LB cultures incubated at 28 °Cfor 91 hours. Five replicate experiments were performed, and appropriate dilutions were streaked on NBTA plates with 50 μg kanamycin per mL to estimate density. In vivo bacterial density was estimated by injecting approximatively 2000 cells (i.e. 20 μL of cultures diluted to 1:1000) in last instar larvae of the lepidopteran *Galleria mellonella* as previously described in (Sicard et al. 2004a). Insect cadavers were homogenized in 100 μLLB after five days of incubation at 28 °C, and appropriate dilutions were streaked on NBTA to estimate density.

We estimated bacteria survival on three isolates (G1#23, G2#25 and G3#9) for which we ran 12 independent agitated LB cultures at 28 °C. Samples were taken in each culture in late exponential phase, early stationary phase and late stationary phase. In each sample, we estimated the total number of cells using a Thoma cell counting chamber and the number of cultivable cells by plating appropriate dilutions of samples on NBTA plates and counting CFU. We then used the proportion of cultivable cells as a proxy for proportion of viable cells, and studied how it varied from exponential phase to early or late stationary phase.

Measuring competitive ability

We measured competitive ability of three fluorescent isolates (G1#23, G2#25 and G3#9) by letting them compete in LB cultures with a non-fluorescent primary

variant (F1V1). Twelve independent late stationary phase cultures of each of the four isolates were used to inoculate these cultures. Before inoculation, cultures of group 2 and 3 variants were 10 fold diluted so that all groups of variants have similar densities. 125 μL of the two competing variants were then mixed, incubated for 120 hours at 28 °C, and plated on NBTA. Pictures of these plates were taken using an Olympus Axiozoom (x7) under fluorescent light (535 nm), so that fluorescent and non-fluorescent colonies could be distinguished. The densities of GFP and non-GFP cells were estimated at the onset of the experiment and after five days of incubation. From these estimates, we could compute a competitive index (CI) as the rate of increase in frequency of GFP bacteria over five days of incubation.

Measuring virulence towards insects

We measured virulence as the time to kill larvae of *G. mellonella*. This was done for five representative isolates of each group (G1#21, G1#23, G1#42, G1#44, G1#51 for group 1, G2#25, G2#29, G2#36, G2#39, G2#40 for group 2 and all members of group 3) and for three injected doses (corresponding to 20 μL of a 1:1000, 1:100 or 1:10 diluted culture). We conducted two replicate experiments and injected each dose four times. Appropriate dilutions of each injected culture were plated on NBTA to estimate the injected dose. We used the automatized procedure described in Parthuisot et al. (2018) to measure time of insect death, and analyzed data with a Cox proportional hazard model, with variation among replicate experiments and variation among isolates within each group considered as a Gaussian random effect. This analysis has been performed using the coxme library (Therneau 2015).

Measuring transmission by *Steinernema carpocapsae*

We associated each of the 34 isolates to the nematode vector *Steinernema carpocapsae* by first infecting *G. mellonella* with aposymbiotic (i.e. deprived of *Xenorhabdus*) nematodes obtained from *S. carpocapsae* strain SK27 and kept alive for a few months at 8 °C in Ringer solution. After 24 hours of incubation at 24 °C in tubes containing 20 nematodes, insects were injected with the isolates. Insect cadavers were subsequently placed in White traps and incubated at 24 °Cuntil new nematodes injective juveniles (IJs) dispersed (Sicard et al. 2004b). In this experiment, two to three independent cultures of each isolate were performed so that a total of 96 independent inocula were tested against three lots of insects, for a total of 288 tests. Inocula of group 1 isolates were less diluted than others (i.e. 1:100 instead of 1:1000) so that $\simeq$ 2000 bacterial cells were injected for all isolates.

For each isolate, we measured the proportion of infections that yielded new IJs and counted the number of living IJs in a 20 μL drop sampled from each emergence. We then estimated the mortality rate of newly emerged IJs by placing four groups of five IJs in Ringer solution, incubating them at 28 °C in the dark for 17 weeks

and counting surviving IJs (Emelianoff et al. 2008). We also quantified the number of bacteria carried per IJs by grinding 20 nematodes for each emergence. IJs had previously been cleaned for ten minutes in 0.4 % bleach and rinsed several times in sterile Ringer solution. They were then placed for ten minutes in TissueLyser II (Qiagen) at 30 Hz with three 3 mm glass beads in order to liberate bacterial symbionts. 100 μL of this suspension were then spread on NBTA plates with kanamycin, and CFU were counted. Finally, we combined these four traits in a single measurement of bacteria transmission, with

$$R_0 = \frac{\beta f e^{-r}}{\nu}$$

where β is parasitic success, f reproductive success, ν IJ mortality rate and r the average number of bacteria carried per IJ (Chapuis et al. 2012). R0 was calculated for each isolate by averaging $\frac{fe^{-r}}{\nu}$ over infections and multiplying this average by the estimate of β.

Variant emergence and reversion *in vitro* and *in vivo*

We studied the emergence of variants in 24 independent static in vitro LB cultures of isolate G1#23 incubated at 28 °C. Samples from each culture were streaked on NBTA plates at day 1, 5 and 7. Five red colonies (when possible) and an equivalent number of blue colonies were then taken from each sample, and their cell size and motility (see above) were measured. On some of these colonies, we also measured antibiotic and haemolytic activities (see above). Following this procedure, we could detect group 2 and group 3 variants rapidly after they emerged. A similar procedure was followed to study reversion to primary form, where we looked for isolates forming blue colonies in prolonged static cultures of group 2 and group 3 variants.

We also followed the emergence of variants after injection of G1#23 in *G. mellonella*. Twenty insects were homogenized after one, two, three, six or ten days of incubation at 28 °C. As for *in vitro* emergence, red and blue colonies were sampled from NBTA plates, and their motility was quantified as described above with two replicate measurements for each colony.

Results

Variants isolates of *X. nematophila* can be classified in three phenotypic groups

Variant isolates of *X. nematophila* are generally classified in two groups. Compared to group 1, group 2 variants cannot adsorb dye, are not motile, do not secrete antibiotics and have weak or no hemolytic and lipolytic activities (Akhurst 1980). Among our 34 isolates, 14 had such characteristics (Figure 1A and Table 1). Remarkably, these group 2 variants also had smaller cells than those of group 1 (Figure 1A),

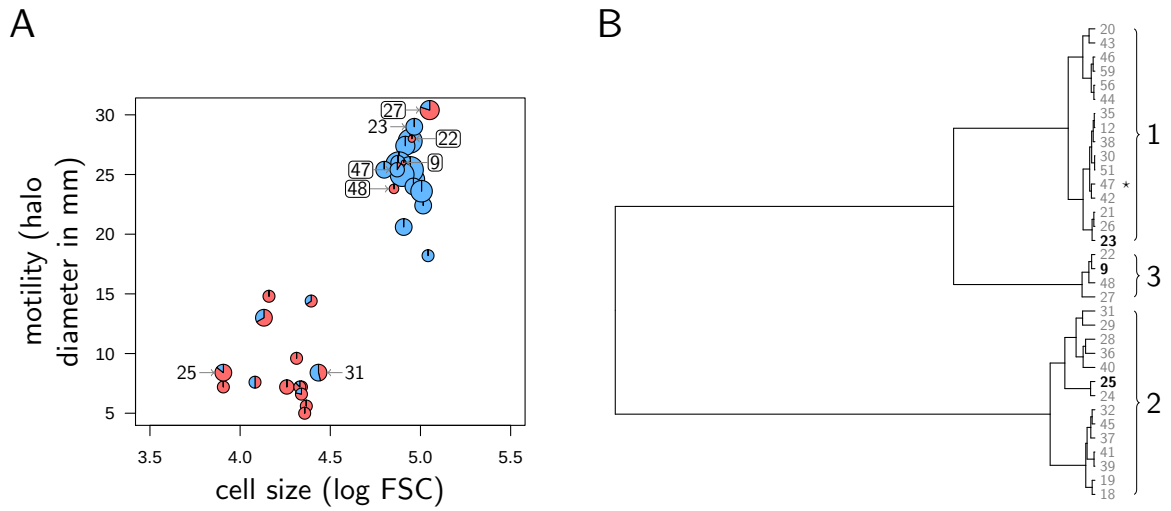

Figure 1: **Variants of *X. nematophila* can be classified in three phenotypic groups**. **A**. Characterization of the 34 isolates: for each isolate average log-FSC, a proxy for cell size, and average motility halo diameter, a measure of cell motility, are represented. The red part of pies chart indicates the proportion of red colonies observed for each isolate on NBTA culture medium, and the pie diameter increases with average measure of antibiotic activity. Boxed numbers identify the five isolates which constitute the third group. Other numbers identify isolates which we will use most often as representative of their group (G1#23 and G2#25 for group 1 and 2 respectively) or that we discuss specifically in text (#31). **B**. Result of a hierarchical clustering analysis (using a UPGMA method) based on log-FSC, motility and proportion of red colonies. The star indicates variant #47, which we added to group 3 but clusters with group 1.

which reports a new characteristic to their set of phenotypes.

The remaining 20 isolates were heterogeneous: they were all motile and had large cells, but 15 of them always formed blue colonies on NBTA while five could form red colonies (Figure 1A). A clustering analysis restricted to these three quantitative phenotypes (motility, cell size and proportion of red colonies) confirmed that four isolates formed a third distinct group of variants (G3#9, G3#22, G3#48 and G3#27, see Figure 1B). We added G3#47 to this group 3, as it clustered with the first group but also formed red colonies. Group 3 variants repeatedly combined some characteristics of group 1 variants (large cells, high motility and high level of lipolytic activity) and others of group 2 variants (low level of antibiotic activity and, most of all, red color colonies on NBTA, see Table 1).

Spontaneous mutations in *lrp* produce the phenotypical difference between group 1 and group 2

Defective Lrp mutants are not motile and have reduced antibiotic and haemolytic activities (Cowles et al. 2007), just like the group 2 isolates of our collection. It is therefore possible that the group 2 variants are *lrp* mutants. To test this hypothesis, we sequenced *lrp* and its promoter region for the 34 isolates of our collection. All group 1 and group 3 isolates had a *lrp* sequence identical to that of the F1 reference genome (Lanois et al. 2013). Conversely, 13 out of the 14 group 2 isolates had one non-synonymous mutation. G2#31 (see Figure 1A) was the sole group 2 isolate with no mutation. Interestingly, G2#31 also stands apart as the group 2 variant with the lowest in vitro growth rate (see Supp. Mat. 2). To discard the possibility that other mutations exist that could be responsible for the phenotype, we performed a complementation by inserting a functional copy of *lrp* in the chromosome of G2#25, which has a SNP in *lrp* codon 120. The complemented variant with group 2 phenotypes showed a full restoration of the phenotypes that are typical of group 1 variants (see supp. Mat. 3).

To validate that these results were not only restricted to our collection, we initiated 24 independent *in vitro* cultures from the group 1 variant G1#23, which we plated every day on NBTA medium. Beyond being an experimental replicate, this experiment allowed us to follow the changes of phenotypes over time (at 1, 5 and 7 days). As expected, all clones initially formed blue colonies and were motile (figure 2A). After five days of incubation, we were able to sample bacteria forming red colonies (figure 2A). They did not differ from other isolates in terms of cell size but had a significantly lower motility and antibiotic activity (Kruskal-Wallis rank sum test $p = 1.41e-04$ and $p = 1.08e-05$ for motility and antibiotic activity respectively, averaged for each culture). After seven days of incubation (figure 2A) bacteria forming red colonies were small non-motile cells while those forming blue colonies were large motile cells (Kruskal-Wallis rank sum test, $p < 2.2e-16$ for both cell size and motility averaged for each culture). Over the seven days of culture, the

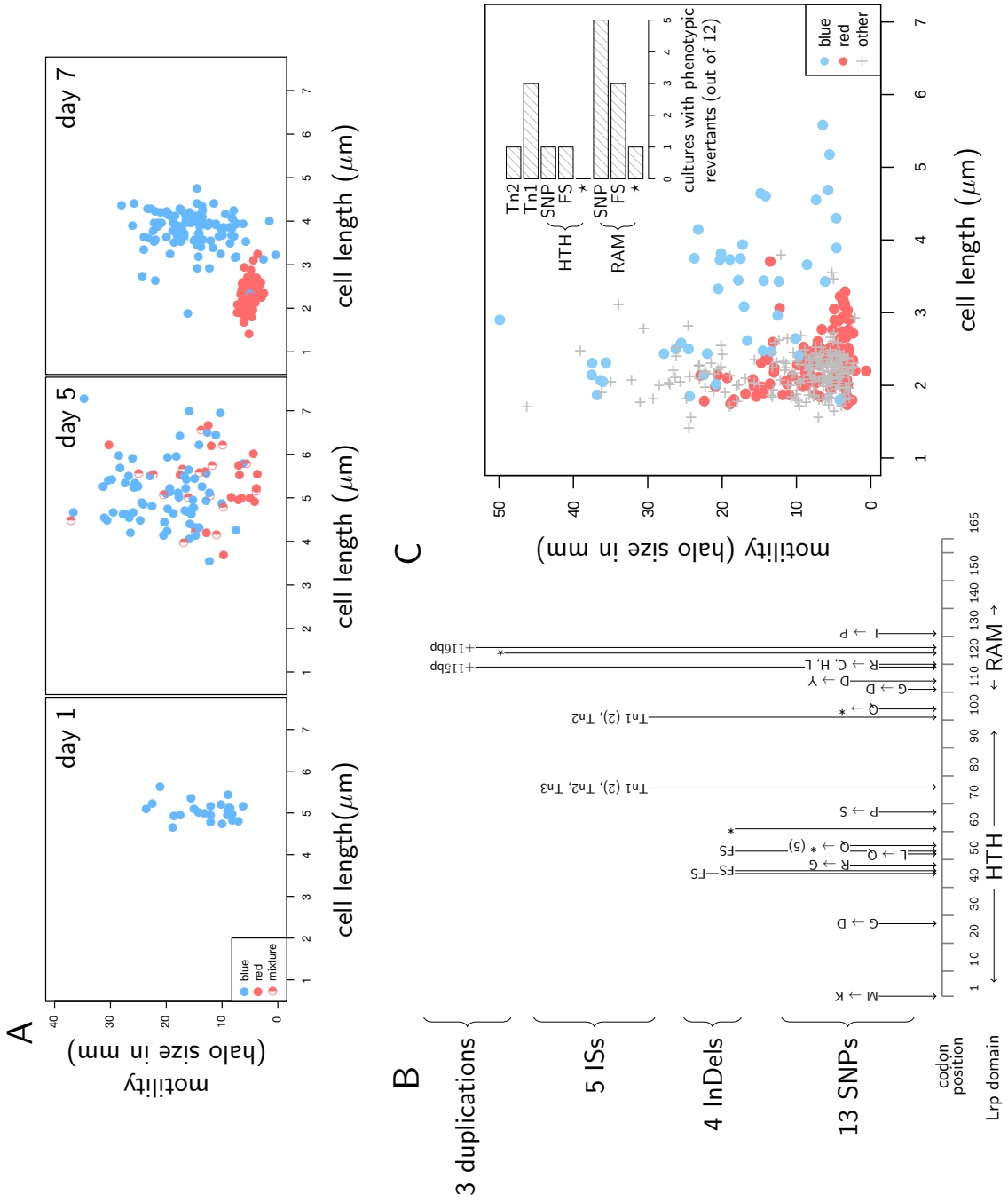

Figure 2: See caption on next page

Figure 2: **Group 2 variants are lrp mutants**. **A-C**. Cell size and motility measured on clones sampled in 24 independent static cultures of variant G1#23. Data are shown after one, five and seven days of incubation. Filled circles (blue or red) represent clones producing only one color of colony, while half-filled circles represent clones producing mixtures of red and blue colonies when streaked on NBTA. **B**. Summary of mutations found in the regulatory gene *lrp*. All the *lrp* mutations we found in group 2 variants were located in the coding sequence, where HTH (Helix-Turn-Helix) is the protein domain that contains the Lrp DNA-binding site and RAM (Regulation of Amino acid Metabolism) is the co-regulator response domain of Lrp. FS stands for frameshift, and stars indicate nonsense mutations which cause the truncation of the translated protein. For SNPs that cause a substitution, the change in amino-acid is given. Tn1, Tn2 and Tn3 are three transposons. Numbers in brackets give the number of independent replicate cultures where a mutation has been observed (one if not indicated). The mutations we found are grouped here in four categories: 3 mutations were tandem duplications of a 26 to 116 bp long fraction of the *lrp* sequence; five mutations were Insertion Sequences (IS) with Tn1, Tn2 and Tn3 being three different transposons which belgon to the group of IS5 insertion sequences, and share the same two insertion points in *lrp*; four mutations were insertion or deletion of few bases (InDels); 13 mutations were single nucleotide polymorphism (SNPs). **C**. Cell size and motility of cells sampled from prolonged culture of 8 different group 2 isolates (one sense mutation, one nonsense mutation and one frameshift in either HTH domain or RAM co-regulator response domain, and two transposon insertions). Points color corresponds to the color of colonies on NBTA medium, gray cross indicating that color is neither blue nor red. Height days after cultures were started, we observed cells that have recovered part of the functions typical of group 1 variants. Many of these reversions, however, are not complete: some revertants have large cells but remain non-motile; other are motile but have small cells or are still red. Inlet: the number of cultures (out of the 12 we performed for each isolate) where blue or haemolytic colonies were observed. HTH and RAM identify isolates which have a mutation in either of the two main active domains of the Lrp protein. As in figure 1C, Tn1 and Tn2 are two distinct insertion sequences, FS stands for frameshift, SNP indicates sense point mutations, and the star corresponds to nonsense mutations.

frequency of red variants increased from 0 % to about 20 %.

We then sequenced *lrp* in clones sampled after eight days of incubation and found non-synonymous mutations in 15 out of the 18 sampled red colonies. Red variants sampled at day five were also *lrp* mutants, although in a smaller proportion (8 out of 14 sequenced clones). Red variants with and without *lrp* mutations had similarly low antibiotic activity (Wilcoxon test: $p = 0.26$) but non-mutated red variants were more motile (Wilcoxon test: $p = 2.66e-3$ and $p = 1.65e-3$ at day 5 and day 8 respectively) and had larger cells at day 8 (Wilcoxon test, $p = 1.65e-3$). They therefore match our definition of group 3 (Table 1). Furthermore, their frequency decreased from day 5 to day 8, which suggests that group 3 variants appeared earlier and were replaced by group 2 variants. This is confirmed by another replicate experiment, where we found red colonies appearing early to be significantly more motile than those appearing late (see supp. Mat. 4).

	Group 1 ($n = 15$)	Group 2 ($n = 14$)	Group 3 ($n = 5$)
dye adsorption	yes	no	no
motility (halo in mm)	24.72±0.71 a	8.73±0.84 b	26.72±1.14 a
antibiotic activity (halo in mm)	17.89±1.38 a	11.35±0.62 b	7.95±3.13 b
hemolytic activity	1 a	0 b	0.4 b
lipolytic activity	0.93 a	0.07 b	1.00 a
cell size ($\log_{10}$ FSC)	4.94±0.02 a	4.24±0.05 b	4.93±0.05 a
lrp mutation	no	yes	no

Table 1: Average phenotypic caracteristics (± standard error) of the three variant groups. Letters indicate significant differences among groups, as determined by a pairwise Wilcoxon test with Holm multiple tests correction. Dye adsorption, haemolytic and lipolytic activities are indicated here as the proportion of variants with high activity and the significance of differences among groups is then tested by a pairwise comparison of proportions. For each group, we indicate whether or not variants carry a non-synonymous mutation in *lrp*. G2#31 is one exception to the rule we indicate here, as it clusters with group 2 variants (Figure 1B) but has no *lrp* mutation.

lrp mutations are diverse and not reversible

Phenotypic variants in *Xenorhabdus* have so far been considered as phase variants (Boemare et al. 1997). If this theory is correct, mutations in *lrp* should be the product of a specific molecular mechanism (van der Woude 2011). Overall, we identified 25 distinct non-synonymous mutations in *lrp* (Figure 2B). 15 of these most probably caused profound alterations to the translated protein: five IS5 insertions and three large duplications totally modified the *lrp* sequence, three InDels caused a frameshift, and one InDel and three SNPs caused nonsense mutations. The remaining 10 mutations are SNPs that changed a single amino acid in Lrp. The mutations we found in group 2 variants are therefore highly diverse, which makes them unlikely to result from a single specific molecular mechanism.

If group 2 variants were phase variants, *lrp* mutations should also be reversible (van der Woude 2011). We tested this by following prolonged static LB cultures of eight group 2 isolates with distinct *lrp* mutations. After 8 days of incubation, we observed cells capable to form blue colonies for most of tested group 2 isolates (Figure 2C). The sequencing of *lrp* revealed that none of these phenotypic reversions was associated with a genetic reversion of the initial mutation. Accordingly, most of these phenotypic reversions were only partial, with blue colonies being composed of either small or weakly motile cells (Figure 2C). This confirms that the *lrp* mutations in group 2 isolates are not produced by a phase variation mechanism. Interestingly, the probability of phenotypic reversion differed among the tested isolates (Figure 2C inlet, glm with binomial error: $\chi^2 = 31.38$, $df = 7$, $p = 5.28e-05$) which suggests that the way phenotypes are restored in *lrp* mutants depends on the precise nature of the mutation. Finally, we never observed revertants in cultures of the group 3 variants G3#9, G3#22 and G3#48.

Group 2 and group 3 variants are under positive selection during prolonged *in vitro* culture

Variants of *X. nematophila* reach high frequency in prolonged *in vitro* cultures, which suggests they are under strong positive selection. To test this, we first estimated bacterial survival during stationary phase (Figure 3A). All three tested variants had the same survival during early stationary phase (Wilcoxon test: $p > 0.07$) but during late stationary phase, G1#23 experienced a ten-fold decrease in survival (Wilcoxon test: $p = 5e-4$) while G2#25 and G3#9 maintained high survival (Wilcoxon test: $p > 0.09$). Group 2 and group 3 variants therefore seem to resist better than group 1 variants to the stressful conditions of late stationary phase explaining why group 2 and group 3 variants reach higher densities than group 1 variants in prolonged culture (see Supp. Mat 2).

We then investigated competitive ability of the same three isolates (Figure 3B). For this purpose, we inoculated G1#23, G2#25 and G3#9 in a LB culture of F1V1, a non-GFP labeled group 1 variant. We then quantified the variation of frequency of GFP labeled variants (Competitive Index, CI) after five days of incubation. G1#23 slightly decreased in frequency (Wilcoxon test: $p = 0.016$) while G2#25 and G3#9, conversely, both increased in frequency (Wilcoxon test: $p = 4.88e-4$ in both cases). G3#9 had an intermediate competitive advantage (Wilcoxon test: $p = 1e-4$) which correlates with the previous observation that group 3 variants had intermediate phenotypes. Altogether, our results suggest that phenotypes of group 2 and group 3 variants have a Growth Advantage in Stationary Phase (GASP, Finkel 2006) and are therefore under positive selection in aged cultures.

Group 2 and group 3 variants also appear inside insects

Akhurst (1980), in its first description of *Xenorhabdus* variants, mentions that they appear *in vitro* but also in insects during infection. To test this in the case of our particular strain of *X. nematophila*, we monitored the appearance of group 2 and group 3 variants after injecting the group 1 isolate G1#23 in *G. mellonella*. As observed *in vitro*, red variants increased in frequency and reached 10 % of the CFUs on average ten days after injection (figure 4A). Red colonies were initially slightly (although non-significantly) more motile than blue colonies, as expected for group 3 variants, but became significantly less motile at both days 3 and 6, as expected for group 2 variants (see figure 4B). Lack of difference at day 10 is most likely explained by the fact that motility decreases in blue cells at that time. Finally, we found that red variants isolated from infected *G. mellonella* were *lrp* mutants when they had group 2 phenotypes, but carried no mutation when they rather had group 3 phenotypes.

The increase in variant frequency in infections (Figure 4A) suggests that the conditions that favor group 2 and 3 variants *in vitro* may also apply inside insects.

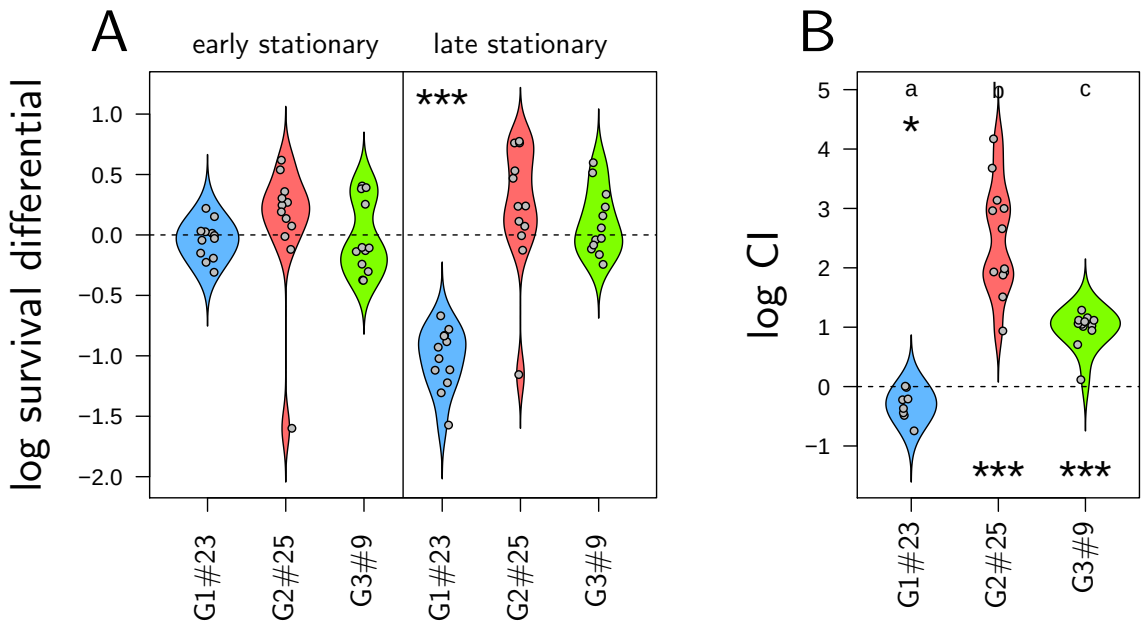

Figure 3: **Group 2 and group 3 variants are under positive selection during prolonged in vitro culture**. **A**. Bacterial survival during early and late stationary phase. We estimated the proportion of living bacteria as the ratio between the total number of cells in a LB culture (estimated with a Thoma cell counting chamber) and the number that can form colonies on NBTA. We contrast here the proportion of living bacteria measured in early or late stationary phase to that measured during exponential phase. Stars indicate significant deviation from zero, as tested by a Wilcoxon test. Variants survival does not significantly vary between early stationary phase and exponential phase. During late stationary phase, G1#23 experiences a ten fold decrease in survival while G2#25 and G3#9 survival does not vary significantly. **B**. Log Competitive Index (CI) of G1#23, G2#25 and G3#9 inoculated in a F1V1 (non-GFP variant from group 1) culture. For each culture CI is estimated as the variation in proportion of GFP-labeled variants. Stars indicate significant deviation from zero, as tested by a Wilcoxon test. As expected, the group 1 GFP-labeled variant G1#23 marginally decrease in frequency when inoculated in the non GFP-labeled group 1 F1V1. Conversely G2#25 and G3#9 both increase in frequency.

	Chisq	df	p
log CFU injected	94.923	1	0.0000
group	17.926	2	1.2805e-04
interaction	4.149	2	0.1256

Table 2: Analysis of survival data. Time to death has been analyzed using a Cox proportional hazard model. The model includes two random effects that account for variation among replicate experiments and variability among variants within each group. Each simple effect (log CFU injected and group) has been tested while the other simple effect was kept in the model. Therefore, the significant effect of group cannot be explained by a difference in inoculum size among groups.

To test this, we measured bacterial densities of the 34 isolates of our collection five days after injection in *G. mellonella* larvae. We found that the density *in vivo* positively correlated with the density variants reached *in vitro* (figure 4C; Kendall correlation: $\tau = 0.40$, $p = 0.7e-4$): the variants that best performed *in vitro* also reached high densities in insects.

Group 2 variants are less virulent and least transmitted

Transmission of *Xenorhabdus* relies in part on their capacity to kill the insect host. We found that all variants retained this capacity, although group 2 variants took slightly more time than others to kill *G. mellonella* (see Figure 5A and Table 2). We then calculated a proxy of bacterial transmission, R_0, which incorporates nematodes parasitic and reproductive success, the number of *Xenorhabdus* bacteria carried per IJ and IJs survival during dispersal (Chapuis et al. 2012). We found that group 1 and group 3 variants had similar transmissions, while group 2 variants had a much lower transmission (Figure 5B, Pairwise Wilcoxon test with Holm correction: $p = 0.14$ and $p = 3.1e-07$ respectively). Reduced transmission in group 2 variants was explained by a lower parasitic success, a reduced nematodes fecundity and an increase in nematodes death rate (Kruskal-Wallis test: $p = 1.11e-5$, $p = 3.40e-5$ and $p = 2.85e-4$ respectively). Isolates that reached the highest *in vivo* (and *in vitro*) loads were the least transmitted (Figure 5B, Kendall's rank correlation: $\tau = -0.43$, $p = 1.946e-4$) although they did reassociate with nematodes (see Figure 5C, Kendall's rank correlation: $\tau = 0.26$, $p = 0.032$). These isolates were not transmitted mostly because they impaired nematodes reproduction (Kendall's rank correlation between f and *in vivo* CFU: $\tau = -0.39$, $p = 8.55e-4$).

As expected, switching occurred during infections. Surprisingly, we found that group 3 variants did revert in the insect, contrary to what we observed *in vitro*, with group 3 infections producing IJs that carry bacteria forming blue colonies. We also found that small proportions of IJs emitted from group 1 infections carried red variants, while a much higher proportion of group 2 infections produced IJs which carried blue revertants (Kruskal-Wallis: $p = 4.74e-3$).

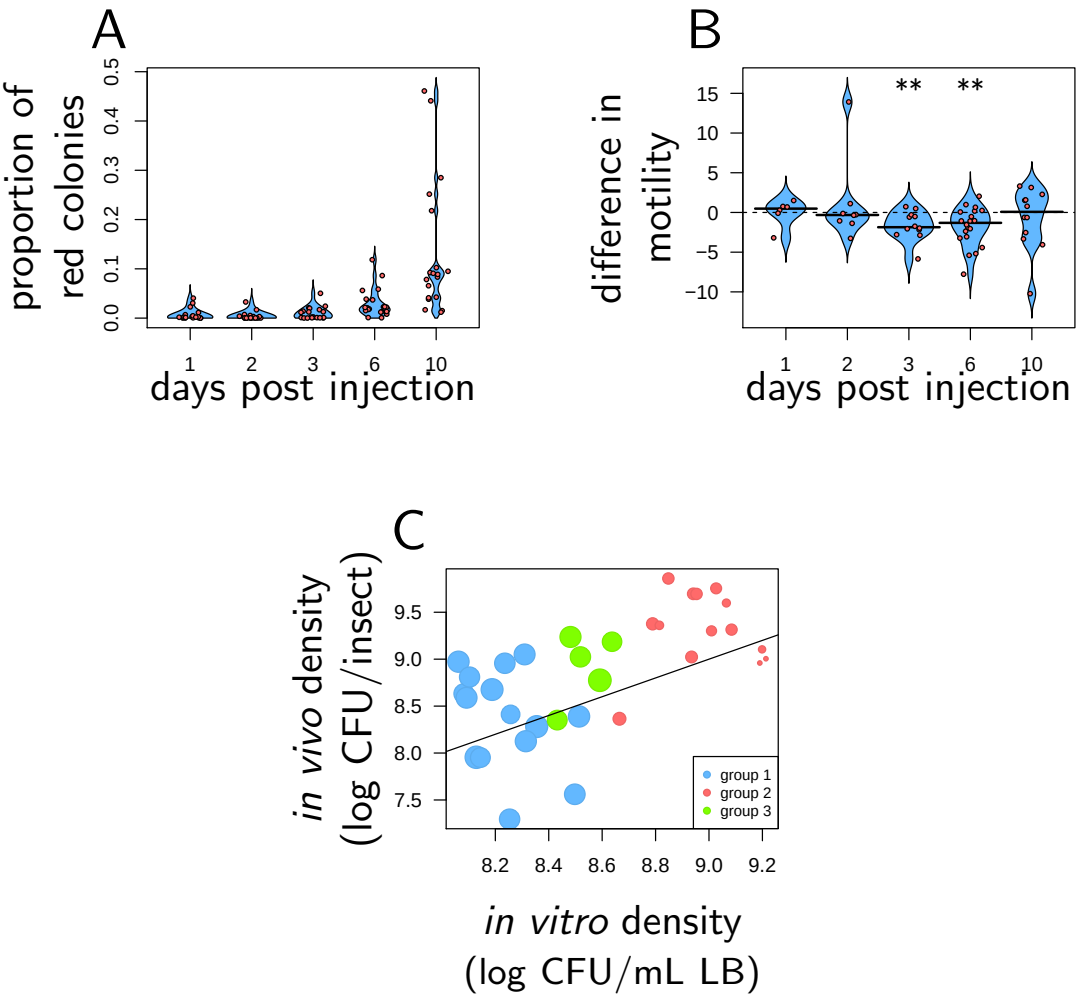

Figure 4: **Group 2 and group 3 variants increase in frequency in the insect during infection**. **A**. Increase in red colonies frequency over the 10 days of an in vivo experiment. **B**. Difference in motility between red and blue cells sampled in insects. Stars indicate that difference in motility significantly deviates from zero, as tested by a Wilcoxon test. Compared to blue isolates, red isolates are initially as motile (as expected for group 3 variants) but becomes less motile at day 3 and 6 (as expected for group 2 variants). **C**. Log CFU per insect (five days after injection, see Materials and Methods) as a function of log CFU per mL in agitated LB culture (after 91 hours of incubation, see Materials and Methods). Each point on a graph corresponds to one of the 34 isolates of the collection, color indicating the group the variant belongs to and point size increasing with cell size (approximated by log-FSC as in Figure 1A). Bacterial density in insects strongly and positively correlates to density in vitro which suggests that traits favored in aged LB cultures are also favored during late infections.

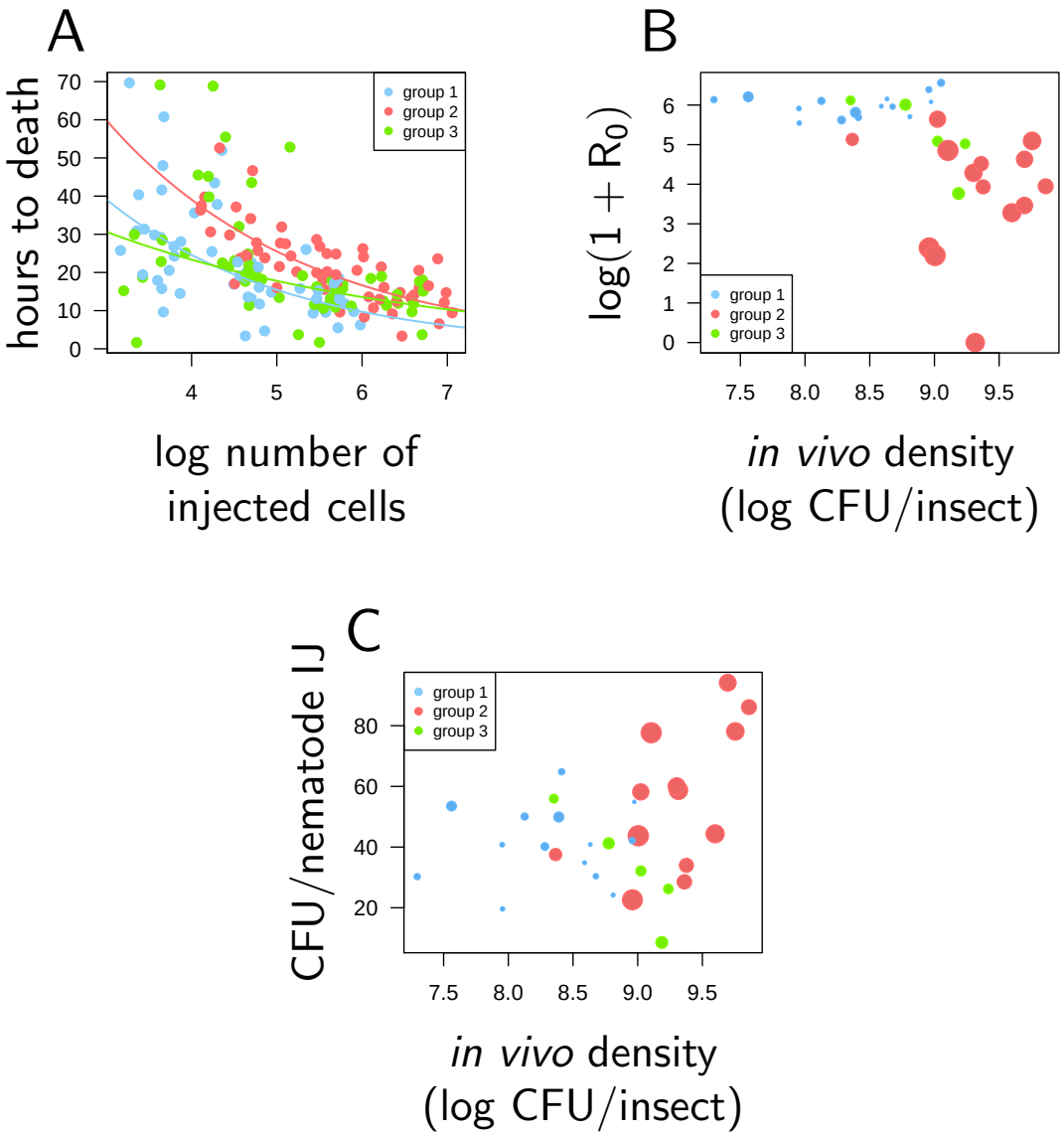

Figure 5: **The most competitive group 2 and group 3 variants are the least transmitted**. **A**. Time to death (hours after injection) for five isolates of each group, as a function of the log-transformed number of injected cells. Each dot represents a single insect, for which the density of inoculum has been estimated so that the number of injected cells could be computed. Curves correspond to the prediction of a survival regression performed on each group of variants. They illustrate the fact that for a given inoculum size group 2 variants take longer to kill the insect. **B**. R0, a measure of *Xenorhabdus* transmission, as a function of the log CFU per insect. This measure of bacteria density is that represented on the y axis of figure 4C. R0 incorporates nematodes parasitic success, reproductive success, survival and the number of bacteria carried on average by a nematode IJ. Each point is one of the 34 isolates, and point size increases with the log number of CFU per mL the isolate reaches on average in LB cultures (the x axis of figure 4C). **C**. Average number of bacteria per nematode IJ, as a function of the log CFU per insect. Again, the measure of bacteria density is that represented on the y axis of figure 4C and point size increases with the average number of CFU per mL reached in LB cultures.

Discussion

Xenorhabdus nematophila secondary variants are characterized by a well known suite of phenotypic traits (inability to adsorb bromothymol blue, reduced motility, reduced antibiotic, haemolytic, lipolytic and proteolytic activities Akhurst 1980; Boemare et al. 1988; Givaudan et al. 1995) to which we added in this work their smaller cell size and the fact that they better survive and reach higher densities in prolonged culture. Earlier studies have demonstrated that group 2 variants share many of these traits with *lrp* defective mutants, and it has therefore been proposed that Lrp was controlling phenotypic variation in *X. nematophila* (Cowles et al. 2007). We demonstrate here that the switch to secondary forms in *Xenorhabdus* is caused by *lrp* mutations. But contrary to what is expected in phase variation (van der Woude 2006), these mutations were of diverse nature and we never observed complete phenotypic reversions involving the restoration of a functional *lrp* sequence. Overall, our data therefore demonstrate that secondary variants of *X. nematophila* are not phase variants, but rather plain *lrp* mutants. The fact that we observed reversion at rates that vary among *lrp* mutants suggests that reversion is achieved through a variety of compensatory mechanisms, probably involving several Lrp regulated genes that are yet to identify.

Surprisingly, we never observed synonymous mutations in *lrp*. This indicates that *lrp* is not a mutational hot-spot and that *lrp* mutants appeared frequently in our experiment because they are under strong positive selection. *lrp* belongs to a family of global regulators which are known to respond to nutrient availability and regulate cell metabolism in case of food shortage (Brinkman et al. 2003; Unoarumhi et al. 2016). Previous work (Richards et al. 2009) and our own *in vitro* measurements (Supp. Mat. 2) demonstrate that *Xenorhabdus lrp* mutants grow slower in rich culture medium, but we found that they survive better and reach ten fold higher loads during prolonged stationary phase. Most importantly, *lrp* mutants did out-compete group 1 variants in aged cultures, a phenotype described as Growth Advantage in Stationary Phase (GASP, Finkel 2006). This interpretation is further strengthened by the fact that one of the first GASP mutants identified was a *lrp* mutant of *Escherichia coli* K-12 (Zinser et al. 2000; Zinser et al. 2004). We therefore propose that secondary variant in *X. nematophila* are GASP mutants which reach high frequency rapidly and repeatedly in late infection because they are then under strong positive selection.

We also documented here the existence of a third class of phenotypic variants, which do not carry *lrp* mutations and share phenotypic characteristics with both group 2 (red colonies, reduced antibiotic activity) and group 1 variants (large and motile cells, lipolytic activity). Interestingly, such a combination of traits has been reported in some variants which Cowles et al. (2007) considered as secondary forms, but might in fact well be similar to our group 3 variants (see Table 2 in Cowles et al. 2007). These variants out-compete group 1 variants and thus display a GASP

phenotype, although weaker than that we measured in group 2 variants. The genetic or epigenetic mechanism responsible for the emergence of this phenotype is yet to be identified.

We showed that variants of group 2 and 3 better resist than group 1 variants to the conditions of *in vitro* late stationary phase. We found indications that the combination of traits that make them more competitive in these conditions is also advantageous in the insect: the variants that reach the highest densities in aged LB cultures also reach the highest loads in late infections. This explains why variants of group 2 and 3 repeatedly appear in insects. This also asks the question of their adaptive value in the natural situation where they interact with their nematode vector. In fact, *lrp* mutants are poorly transmitted, which cannot be explained by a deficiency in re-association with nematodes, as we found that IJs emitted from group 2 infections carried as much bacteria as those from group 1 infections. This observation contradicts the prediction of Cao et al. (2017) but supports previous findings by Sicard et al. (2004a). Low transmission of group 2 rather comes from a sharp decrease in nematodes reproduction, which is in agreement with many earlier experimental results (e.g. Cowles et al. 2007; Cao et al. 2017) and corresponds to the well established detrimental effect of secondary variants in mass production of *S. carpocapsae* (e.g. Hirao et al. 2009). We also found that IJs emitted from group 2 infections have lower survival during dispersal, which is probably yet another indication that infections initiated with *lrp* mutants constitute an unfavorable environment for *S. carpocapsae*, consistently with Cao et al. (2017).

Chapuis et al. (2012) demonstrated that death rate of IJs increases with the number of *X. nematophila* cells they carry. As a result, IJs survival during dispersal trades off against their capacity to initiate a new infection. Here we show that variants that reach higher loads in late infections, are the least transmitted by nematodes (figure 4C). This can be understood as yet another trade-off, distinct from that demonstrated by Chapuis et al. (2012), where traits permitting high loads in infection decrease nematodes reproduction. In *E. coli*, *lrp* mutants are thought to be better adapted to late stationary phase in part because they scavenge some of the amino acids they need, instead of producing them (Zinser et al. 2000; Zinser et al. 2004). In *X. nematophila*, where *lrp* also controls the production of exo-enzymes which are known to support nematode reproduction (Cowles et al. 2007; Hussa et al. 2015; Cao et al. 2017; Casanova-Torres et al. 2017; Engel et al. 2017) this advantage would come at the cost of a reduction in transmission.

Xenorhabdus must persist from one to two weeks in an insect cadaver, before being transmitted. At that time, our results suggest that the population of pathogens is composed of a variety of GASP phenotypes. Similar situations could probably arise in other pathogens that stay for many generations inside their host (e.g. Poussier et al. 2003; Simsek et al. 2018) and regulators controlling metabolism, such as *lrp*, could then be the targets of a form of diversifying selection (Simsek et al.

2018). GASP mutants, which we found can impact transmission, may therefore influence the evolution of pathogens that form long lasting infections.

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Competing interest

The authors declare that they have no competing financial interests in relation to the work described in this paper.

Supplementary material

Supplementary material can be downloaded here: `https://dl.univ-tlse3.fr/filez/vw6pl`

References

Akhurst, R. (1980). "Morphological and functional dimorphism in *Xenorhabdus* spp bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*". *J. Gen. Microbiol.* 121, pp. 303–309.

Bayliss, C. D. (2009). "Determinants of phase variation rate and the fitness implications of differing rates for bacterial pathogens and commensals". *FEMS Microbiol. Rev.* 33.3, pp. 504–520.

Boemare, N., J. Thaler, and A. Lanois (1997). "Simple bacteriological tests for phenotypic characterization of *Xenorhabdus* and *Photorhabdus* phase variants". *Symbiosis* 22, pp. 167–175.

Boemare, N. and R. Akhurst (1988). "Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp.(Enterobacteriaceae)". *J. Gen. Microbiol.* 134.3, pp. 751–761.

Brinkman, A. B., T. J. G. Ettema, W. M. De Vos, and J. Van Der Oost (2003). "The Lrp family of transcriptional regulators". *Mol. Microbiol.* 48.2, pp. 287–294.

Cao, M., T. Patel, T. Rickman, H. Goodrich-Blair, and E. A. Hussa (2017). "High levels of the *Xenorhabdus nematophila* transcription factor Lrp promote mutualism with the *Steinernema carpocapsae* nematode host". *Appl. Environ. Microbiol.* 83.12. Ed. by A. J. McBain, AEM–00276.

Casanova-Torres, Á. M., U. Shokal, N. Morag, I. Eleftherianos, and H. Goodrich-Blair (2017). "The global transcription factor Lrp is both essential for and inhibitory to *Xenorhabdus nematophila* insecticidal activity". *Appl. Environ. Microbiol.* 83.12. Ed. by H. L. Drake.

Chapuis, E., A. Arnal, and J.-B. Ferdy (2012). "Trade-offs shape the evolution of the vector-borne insect pathogen *Xenorhabdus nematophila*". *P. Roy. Soc. B-Biol. Sci.* 279.1738, pp. 2672–2680.

Cowles, K. N., C. E. Cowles, G. R. Richards, E. C. Martens, and H. Goodrich-Blair (2007). "The global regulator Lrp contributes to mutualism, pathogenesis and phenotypic variation in the bacterium *Xenorhabdus nematophila*". *Cell. Microbiol.* 9.5, pp. 1311–1323.

Diard, M., V. Garcia, L. Maier, M. N. Remus-Emsermann, R. R. Regoes, M. Ackermann, and W.-D. Hardt (2013). "Stabilization of cooperative virulence by the expression of an avirulent phenotype". *Nature* 494.7437, pp. 353–356.

Didelot, X., A. S. Walker, T. E. Peto, D. W. Crook, and D. J. Wilson (2016). "Within-host evolution of bacterial pathogens". *Nat. Rev. Microbiol.* 14.3, p. 150.

Emelianoff, V., E. Chapuis, N. Le Brun, M. Chiral, C. Moulia, and J.-B. Ferdy (2008). "A survival-reproduction trade-off in entomopathogenic nematodes mediated by their bacterial symbionts". *Evolution* 62.4, pp. 932–942.

Engel, Y., C. Windhorst, X. Lu, H. Goodrich-Blair, and H. B. Bode (2017). "The Global Regulators Lrp, LeuO, and HexA Control Secondary Metabolism in Entomopathogenic Bacteria". *Front. Microbiol.* 8, p. 209.

Finkel, S. E. (2006). "Long-term survival during stationary phase: evolution and the GASP phenotype". *Nat. Rev. Microbiol.* 4.2, p. 113.

Forst, S. and B. Boylan (2002). "Characterization of the pleiotropic phenotype of an ompR strain of *Xenorhabdus nematophila*". *Antonie van Leeuwenhoek* 81.1, p. 43.

Givaudan, A., S. Baghdiguian, A. Lanois, and N. Boemare (1995). "Swarming and swimming changes concomitant with phase variation in *Xenorhabdus nematophilus*." *Appl. Environ. Microbiol.* 61.4, pp. 1408–1413.

Givaudan, A. and A. Lanois (2000). "flhDC, the flagellar master operon of *Xenorhabdus nematophilus*: requirement for motility, lipolysis, extracellu-

lar hemolysis, and full virulence in insects". *J Bacteriol.* 182.1, pp. 107–115.

Goodrich-Blair, H. (2007). "They've got a ticket to ride: *Xenorhabdus nematophila-Steinernema carpocapsae* symbiosis". *Curr. Opin. Microbiol.* 10.3, pp. 225–230.

Hirao, A. and R.-U. Ehlers (2009). "Influence of cell density and phase variants of bacterial symbionts (*Xenorhabdus* spp.) on dauer juvenile recovery and development of biocontrol nematodes *Steinernema carpocapsae* and *S. feltiae* (Nematoda: Rhabditida)". *Applied Microbiology and Biotechnology* 84.1, pp. 77–85.

Huber, W., V. J. Carey, R. Gentleman, S. Anders, M. Carlson, B. S. Carvalho, H. C. Bravo, S. Davis, L. Gatto, T. Girke, et al. (2015). "Orchestrating high-throughput genomic analysis with Bioconductor". *Nature methods* 12.2, p. 115.

Hussa, E. A., Á. M. Casanova-Torres, and H. Goodrich-Blair (2015). "The global transcription factor Lrp controls virulence modulation in *Xenorhabdus nematophila*". *J Bacteriol.* JB–00272.

Lanois, A., J.-C. Ogier, J. Gouzy, C. Laroui, Z. Rouy, A. Givaudan, and S. Gaudriault (2013). "Draft genome sequence and annotation of the entomopathogenic bacterium *Xenorhabdus nematophila* strain F1". *Microbiol.Res. Ann.* 1.3.

Markussen, T., R. L. Marvig, M. Gómez-Lozano, K. Aanæs, A. E. Burleigh, N. Høiby, H. K. Johansen, S. Molin, and L. Jelsbak (2014). "Environmental heterogeneity drives within-host diversification and evolution of *Pseudomonas aeruginosa*". *mBio* 5.5. Ed. by R. Kolter.

Parthuisot, N., J. Rouquette, and J.-B. Ferdy (2018). "A high-throughput technique to quantify bacterial pathogens' virulence on the insect model *Galleria mellonella*". *J. Microbiol. Methods* 152, pp. 69–72.

Poussier, S., P. Thoquet, D. Trigalet-Demery, S. Barthet, D. Meyer, M. Arlat, and A. Trigalet (2003). "Host plant-dependent phenotypic reversion of Ralstonia solanacearum from non-pathogenic to pathogenic forms via alterations in the phcA gene". *Molecular Microbiology* 49.4, pp. 991–1003.

Richards, G. R. and H. Goodrich-Blair (2009). "Masters of conquest and pillage: *Xenorhabdus nematophila* global regulators control transitions from virulence to nutrient acquisition". *Cell. Microbiol.* 11.7, pp. 1025–1033.

Schneider, C. A., W. S. Rasband, and K. W. Eliceiri (2012). "NIH Image to ImageJ: 25 years of image analysis". *Nature methods* 9.7, p. 671.

Sicard, M., K. Brugirard-Ricaud, S. Pagès, A. Lanois, N. E. Boemare, M. Brehélin, and A. Givaudan (2004a). "Stages of infection during the tripar-

tite interaction between *Xenorhabdus nematophila*, its nematode vector, and insect hosts". *Appl. Environ. Microbiol.* 70.11, pp. 6473–6480.

Sicard, M., J.-B. Ferdy, N. Le Brun, B. Godelle, N. Boemare, and C. Moulia (2004b). "When mutualists are pathogens: an experimental study of the symbioses between *Steinernema* (entomopathogenic nematodes) and *Xenorhabdus* (bacteria)". *J. Evolution. Biol.* 17.5, pp. 985–993.

Simsek, E. and M. Kim (2018). "The emergence of metabolic heterogeneity and diverse growth responses in isogenic bacterial cells". *The ISME Journal* 12, pp. 11998–1209.

Therneau, T. M. (2015). *coxme: Mixed Effects Cox Models*. R package version 2.2-4.

Unoarumhi, Y., R. M. Blumenthal, and J. S. Matson (2016). "Evolution of a global regulator: Lrp in four orders of γ-Proteobacteria". *BMC Evol. Biol.* 16.1, p. 111.

van der Woude, M. W. (2006). "Re-examining the role and random nature of phase variation". *FEMS Microbiol. Lett.* 254, pp. 190–197.

— (2011). "Phase variation: how to create and coordinate population diversity". *Curr. Opin. Microbiol.* 14.2, pp. 205–211.

van der Woude, M. W. and A. J. Bäumler (2004). "Phase and antigenic variation in bacteria". *Clin. Microbiol. Rev.* 17.3, pp. 581–611.

Vigneux, F., R. Zumbihl, G. Jubelin, C. Ribeiro, J. Poncet, S. Baghdiguian, A. Givaudan, and M. Brehélin (2007). "The xaxAB genes encoding a new apoptotic toxin from the insect pathogen *Xenorhabdus nematophila* are present in plant and human pathogens". *J. Biol. Chem.* 282.13, pp. 9571–9580.

Volgyi, A., A. Fodor, A. Szentirmai, and S. Forst (1998). "Phase variation in *Xenorhabdus nematophilus*". *Appl. Environ. Microbiol.* 64.4, pp. 1188–1193.

Young, B. C., C.-H. Wu, N. C. Gordon, K. Cole, J. R. Price, E. Liu, et al. (2017). "Severe infections emerge from commensal bacteria by adaptive evolution". *eLife* 6. Ed. by M. T. Holden, e30637.

Zhang, J.-R., J. M. Hardham, A. G. Barbour, and S. J. Norris (1997). "Antigenic variation in Lyme disease Borreliae by promiscuous recombination of VMP-like sequence cassettes". *Cell* 89.2, pp. 275–285.

Zinser, E. R. and R. Kolter (2000). "Prolonged stationary-phase incubation selects for *lrp* mutations in *Escherichia coli* K-12". *Journal of Bacteriology* 182.15, pp. 4361–4365.

Zinser, E. R. and R. Kolter (2004). "*Escherichia coli* evolution during stationary phase". *Res. Microbiol.* 155.5, pp. 328–336.

Project of paper:

Mixture of phenotypic variants inside infections by *Xenorhabdus nematophila*

Marine Cambon[1], Nathalie Parthuisot[1], Sylvie Pagès[2], Quentin Gaertner[1], Alain Givaudan[2] and Jean-Baptiste Ferdy[1]

[1]Évolution et Diversité Biologique, CNRS-UPS-IRD, Université Paul Sabatier - Toulouse

[2]DGIMI - INRA-Université Montpellier 2 - Montpellier

1 Introduction

As most pathogens, *Xenorhabdus nematophila* does evolve inside its host, during the course of infection (Didelot et al. 2016). In a previous work, we showed that this evolution generates a diversity of phenotypes, that we shall hereafter call variants. We showed that some of these variants are in fact plain mutants, the most frequently identified variants bearing non-synonymous mutations in the gene encoding for the master regulator Lrp which controls part of *X. nematophila* metabolism. These *lrp* mutants do not produce many of the secondary metabolites that have been shown to play a role in the degradation of the host tissues and interactions with microbial competitors. We also found that these variants all have in common that they better resist to the harsh conditions of late stationary phase of growth and have therefore an adaptive value in prolonged *in vitro* cultures, but also most probably inside the insect host.

These GASP variants (Growth Advantage in Stationary Phase, Finkel 2006), which do not express some of the costly functions involved in the symbiosis with their nematode vector (Cowles et al. 2007), could reasonably be considered as cheaters. But, we showed that all variants were able to kill the insect host and to re-associate with nematodes, although transmission was highly reduced with *lrp* mutants compared to other variants. Determining whether these variants are cheaters or not, i.e. understanding their impact on the functioning of the symbiosis with the nematode, requires that we study situations where several variants interact inside a single insect. Cheaters are indeed individuals

which reach high fitness because they do not contribute to public goods. Transposed to the case of *X. nematophila*, this would mean that the appearance of these cheaters during the course of an infection should deplete the transmission of all *X. nematophila* cells, being variants or not.

In the most extreme situation, cheaters could be so competitive that they eventually exclude other *X. nematophila* from the infection. This could lead to the complete failure of transmission, and therefore to the destabilization of the symbiosis. To understand the evolutionary consequences of the existence of these variants, we thus need to test whether they can dominate the *X. nematophila* population during infection, or instead coexist and form an heterogeneous population.

In the present study, we first studied how different variants compete with each other in various *in vitro* conditions. We showed in particular that non agitated cultures may promote coexistence of different variants within the population, because competition in this condition is intransitive. We then quantified *X. nematophila* transmission by its vector *S. carpocapsae* when infections are composed either of a single variant type or of mixtures of variants. We confirmed that *lrp* mutants can re-associate with *S. carpocapsae* but are badly transmitted because they impede its reproduction. We showed, however, that the transmission of these mutants can be rescued when other variants (with no *lrp* mutation) are co-injected. The results presented here are still at the stage of preliminary analysis, and more work needs to be done on these data. The main idea of these first results however, is that infections of *X. nematophila* can be mixtures of different variants, that nematode reproduction is ensured as soon as a *lrp* non-mutant variant is present, and that re-association with *S. carpocapsae* is not specific enough to filter out these variants.

2 Materials and Methods

2.1 *In vitro* competitions

Competitions between fluorescent (GFP) and non-fluorescent (non-GFP) variants were performed for each of the three groups. One GFP-variant for each group (i.e. V1, V2, V3) was chosen among the collection of variant described in Cambon et. al (submitted, see page 19). These variants originated from a modified strain that expresses GFP, and thus can be distinguished because it forms fluorescent colonies (Sicard et al. 2004). Mixtures of GFP and non-GFP variants (F1V1, F1V2, F1V3) were incubated for 120 hours at 28°C and subsequently spread on NBTA plates, so that red and blue colonies can be distinguished. Pictures of these plates were taken using an Olympus Axiozoom (×7), both under natural light and under fluorescent light (535nm), so that fluorescent and non-fluorescent colonies could also be distinguished. The number of cells from each variant was thus estimated after 120 hours of competition. Table 1 summarizes the number of

competitions we have studied for each combination of variants and for culture condition (see below).

	F1V1	F1V2	F1V3
V1#23	8	12	12
V2#25	12	8	12
V3#9	12	12	8

Table 1: Number of replicate competitions for each combination of GFP (V1#23, V2#25 and V3#9) and non-GFP variants (F1V1, F1V2 and F1V3). Each replicate was studied in four different culture conditions: Limiting Resources (LR) or Non-Limiting Resources (NLR), with agitation (A) or without agitation (NA). The whole experiment therefore represents a total of 384 independent competition experiments.

Each competition was performed as follows: cryo-conserved clones of the six variants were first grown for 48 hours at 28°C on NBTA plates, with Kanamycin (20 μg/mL) added for the three GFP-labelled variants that carry a Kanamicin resistance gene on plasmid, together with the GFP gene. One colony for each variant was then suspended in 2mL LB and incubated at 28°C for 8 hours with agitation (160 rpm). The six precultures obtained this way (one per variant) were rinsed and each of them served to inoculate 12 replicate cultures. A 100 fold dilution was applied to group 2 and 3 variants; a 50 fold dilution was applied to group 1 variants to compensate for differences in cell density. The resulting 72 cultures represented each a total volume of 1mL, that we placed in 15mL culture tubes. These tubes were incubated for 17 hours (overnight) at 28°C with agitation (160 rpm). Cultures had by then reached late stationary phase and were used to inoculate the competition experiments.

Before inoculation, cultures of group 2 and 3 variants were 10 fold diluted so that all groups of variants have similar densities. In a first set of treatments (Limiting Resources, LR) 125 μL of the two competing variants were mixed, with no additional dilution. Initial conditions were therefore close to that of the late stationary cultures that served to make the inoculums. Two culture plates were produced with such initial conditions: one was incubated with agitation and the other without agitation. In a second set of treatments, all cultures were first a 100 fold diluted, and 25 μL of each diluted culture were mixed with 200 μL of LB. In these treatments, bacterial density was initially low and the competing variants did initially go through exponential phase. Again, two culture plates were produced for this condition, one being incubated with agitation and the other without.

The densities of GFP and non-GFP cells were estimated from counts of Colony Forming Units (CFU) at the onset the experiment and after three and five days of incubation. From these estimates, we could compute a competitive index (CI) as the rate of increase in frequency of GFP bacteria over three or five days of incubation. Data of day 3 have not been analyzed yet, and the following results only concern day 5.

2.2 *In vivo* co-infections

2.2.1 Re-association of bacteria with nematodes

In order to evaluate the impact of a mixture of variants initiating the infection, we measured the transmission R_0 for five GFP variants from each of the three groups (V1#21, #23, #42, #44, #51, V2#25, #29, #36, #39, #40, V3#2, #22, #27, #47, #48), when injected in mixture with non-GFP clones (F1V1, F1V2 and F1V3). To do so, we used the experimental procedure described in Cambon et al. (submitted, see page 19). Briefly, we prepared cultures of GFP and non-GFP variants, and mixed them in 50:50 v/v ratio. Two replicate of each variant combination were made, with independent cultures. To obtain an equivalent number of cells of each group in the mixture, we diluted 10 times more the cultures of variants from group 2 and 3, as they reach higher densities in stationary phase than variants from group 1.

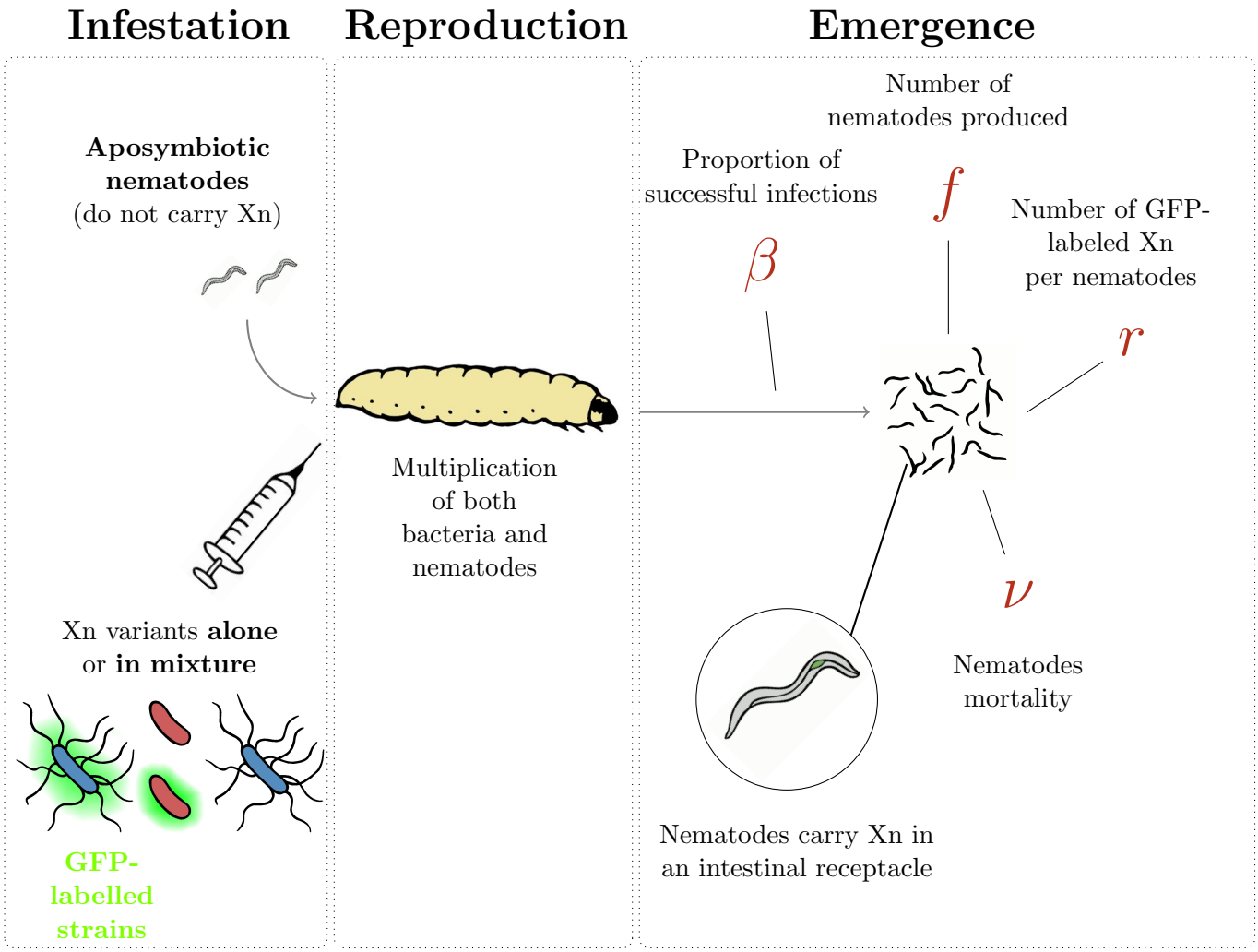

Figure 1: Experimental design to measure transmission R_0 of GFP variants, when injected alone or in mixture with non-GFP variants. Xn: *X. nematophila*

Each mixed inoculum was then injected in three *G. mellonella* previously infected

by 20 aposymbiotic *S. carpocapsae* (i.e. nematodes deprived of their *X. nematophila* symbiont). The same inoculum was also injected in one uninfected *G. mellonella*. Infected insects were placed at 24 °C and IJ emergence from insects was followed each day. For each combination of variants, we measured the proportion of insects that yielded new IJs (parasitic success), estimated the number of IJs produced (reproductive success), and the mean number of *X. nematophila* cells carried by these IJs (retention) (see Cambon et al. submitted, (page 19) and Figure 1 for more details on the experimental procedure). We controlled inoculum size, and measured retention by plating inoculum or ground IJs on NBTA medium and taking pictures of the plates under fluorescent light and natural light as described above. This allows to distinguish GFP and non-GFP variants. However, some other bacteria were found in emerging IJs, and had a very similar shape and color when plated on NBTA plates to that of variants from group 2 of *X. nematophila*. Thus, we focused on GFP variants to be able to accurately distinguish them from other bacteria, and thus specifically measured their transmission. This implies that the R_0 values that are shown do not represent the total transmission of *X. nematophila* after infection, but rather the transmission of the GFP variant.

We estimated the survival rate of emerging IJs after a short period of desiccation. To do so, 10 nematodes from each emergence were placed in 40 μL of Ringer solution in 96 wells plate. Two replicates of each plate were made. The plates were placed at 28 °C without cover for 3 hours to let the Ringer solution evaporate. We took care of randomizing treatment positions in the plate to prevent confounding effects between treatment and evaporation which is not perfectly homogeneous in the plate. All wells were then refilled with 150 μL of distilled water. Plates were placed 24 hours at 28 °C, followed by 48 hours at room temperature. Dead and alive IJs of each well were finally counted to estimate a mortality rate.

Insects that were not previously infected by aposymbiotic nematodes were ground 4 days after infection to estimate the proportion of each variant inside insect. However, these data are not analyzed yet.

The same experimental procedure and R_0 calculation was also performed initiating infection with each fluorescent variant alone.

2.2.2 Estimation of transmission R_0 using a transmission model

The vector parasitic success, reproductive success and mortality rate, as well as the number of bacteria carried by nematodes were combined to estimate the bacterial transmission. To do so, we used the transmission model described in Chapuis et al. (2012) which reproduces the bacteria life cycle. According to this model, the bacterial transmission can be estimated as :

$$R_0 \simeq \frac{\beta f e^{-r}}{\nu} \tag{1}$$

where β is the proportion of successful infection (i.e. leading to nematode emergence), f is the number of nematode produced, ν is the mortality rate of nematodes and r the average number of bacteria carried by nematodes for each infection, assumed to follow a Poisson distribution.

We estimated f, ν and r for each infection (Figure 1). R_0 was then calculated for each variant combination by averaging $\frac{fe^{-r}}{\nu}$ over the different infections with the same combination, and multiplying this quantity by β the proportion of successful infection for this combination.

3 Results

3.1 Group 2 variants are often the most competitive *in vitro*, but competition can be intransitive in non agitated cultures

We measured the *in vitro* competitive advantage of variants by quantifying their change in frequency in different conditions of culture. Changes in frequency in GFP-labelled variants depended on both which non-GFP variant they competed with, and which culture condition they experienced (Figure 2). Overall, the variants from group 2 (V2#25 and F1V2) often out-competed other variants. When resources were non-limiting (Figure 2A and 2B), notably, V2#25 always had CIs positive and higher than those of any other variants.

Within the five days of incubation, phenotype switch occurred in populations of group 1 variants, which then produced cells forming red colonies on NBTA culture medium. This switch of phenotype increased the competitivity of group 1 variants against group 2: CI of V1#23 increases with proportion of GFP-labelled variants forming red colonies when cultures are non-agitated and resources are limiting (figure 3A); symmetrically, the CI of V2#25 decreases with the proportion of non-GFP variants forming red colonies when resources are limiting (figure 3B). Switching therefore seems to increase the competitivity of group 1 variants that compete with group 2 variants. This result is yet another confirmation that group 2 variants are positively selected during prolonged culture. In the case of non-GFP labelled V1, non fluorescent clones forming red colonies could originate from V2#25 or V3#9 that have lost their plasmid. We found this to be unlikely as switching occurred at comparable rates in V1 and V1#23 in most cases (the non-GFP group 1 strain having higher rates in three out of the twelve conditions that include group 1 variants).

Group 2 variants are not always advantaged, though. This is particularly clear when resources are limiting and in the absence of agitation (Figure 2A). GFP-labelled V2 is then still most competitive when mixed with non-GFP V1, but it performs worse than GFP-labelled V3 when facing non-GFP V2 and does not increase in frequency when competing

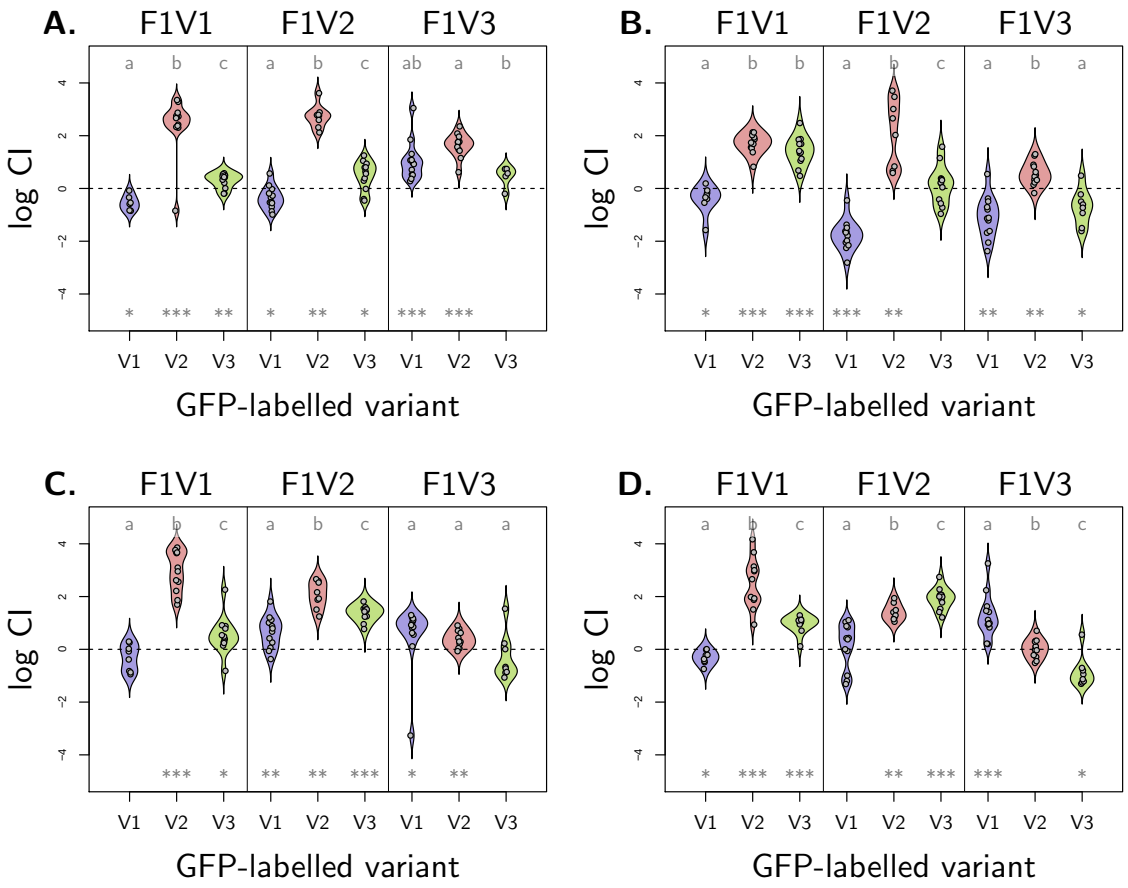

Figure 2: **Log-transformed Competitive Index (CI) of GFP-labelled variants** (V1, V2 and V3) depending on which non fluorescent variant (F1V1, F1V2 and F1V3) they compete with. Each point corresponds to one replicate competition experiment. A value of zero indicates that the frequency of GFP-labelled variants has not varied during incubation; a value of $+1$ (-1) means that frequency has been multiplied (divided) by 10. Each graphic corresponds to one treatment: **A.** limiting resources, non-agitated culture; **B.** non-limiting resources, agitated culture; **C.** limiting resources, agitated culture; **D.** non-limiting resources, non-agitated culture. Stars indicate CI which significantly deviate from zero (*, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001), as tested by a Wilcoxon rank test. Letters indicate GFP variants which significantly differ from each other. This has been tested by a pairwise Wilcoxon test, applying Holm correction for multiple tests (with significance level fixed to 0.01).

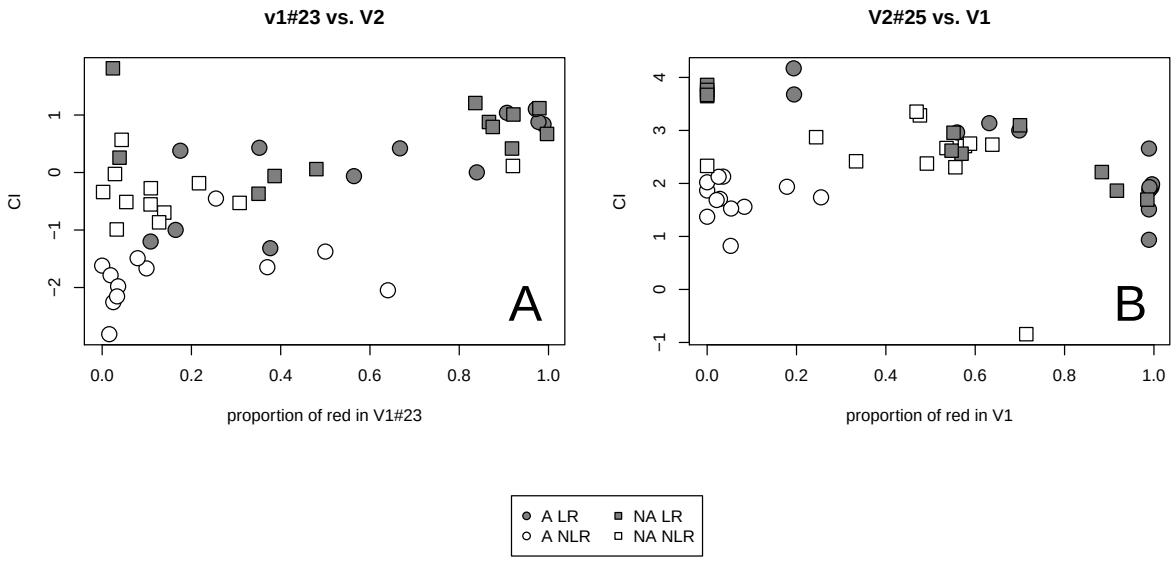

Figure 3: A. Log-transformed Competitive Index (CI) of GFP-labelled variant V1#23 competing with non-GFP V2, as a function of the proportion of red colonies we found in V1#23. CI significantly increases with the proportion of red colonies in non agitated cultures with limiting resources (Kendall rank correlation: $T = 51$, $p = 0.014$ $\tau = 0.54$). In all other treatments, CI is not significantly linked with that proportion (Kendall rank correlation: $p > 0.15$) B. Log-transformed Competitive Index (CI) of GFP-labelled variant V1#25 as a function of the proportion of red colonies we found in the competing non-GFP V1. CI of V1#25 is negatively linked to the proportion of red colonies in V2 when resources are limiting (Kendall rank correlation: $p = 0.031$ and $p = 6.07e - 4$ for non-agitated and agitated cultures respectively). The two quantities are non linked when resources are non limiting (Kendall rank correlation: $p > 0.63$).

with non-GFP V3. In summary, our data suggest that in prolonged and non-agitated cultures group 1 variants can be invaded by group 2 variants, but that group 2 variants can in turn be invaded by group 3 variants, while finally group 3 variants can be invaded by group 1 variants. Therefore, none of the three groups of variants can dominate all other groups: competition seems to be intransitive, as in the classical Rock-Paper-Scissor (RPS) game, which should permit the coexistence of the three groups inside the infection (Kerr et al. 2002).

3.2 Co-inoculation and infestation

We previously showed that when group 2 variants initiate the infection, their transmission is reduced compared to group 1 and 3, because nematodes do not reproduce well. In the present experiment R_0 is strongly correlated to the number of IJs nematodes produced (Spearman's correlation, rho = 0.72, p-value = 1.44e-10) which confirms that nematode reproduction is the main limiting factor of *X. nematophila* transmission. Our results also confirm that GFP-labelled group 2 variant have a much lower R_0 than other variants

when injected alone (Figure 4, V2 alone) or with another group 2 variant (Figure 4, V2 and F1V2). Conversely, they had a R_0 comparable to that of group 1 and 3 variants when co-injected with F1V1 and F1V3 (Figure 4, V2 and F1V1 or F1V3). This suggests that group 2 variants have no impact on nematode reproduction and on *X. nematophila* transmission as long as group 1 (or group 3) variants are present during the early stages of infection. A confirmation of this is that GFP-labelled group 1 and group 3 variants always have high R_0, whatever variant they are injected with (Figure 4).

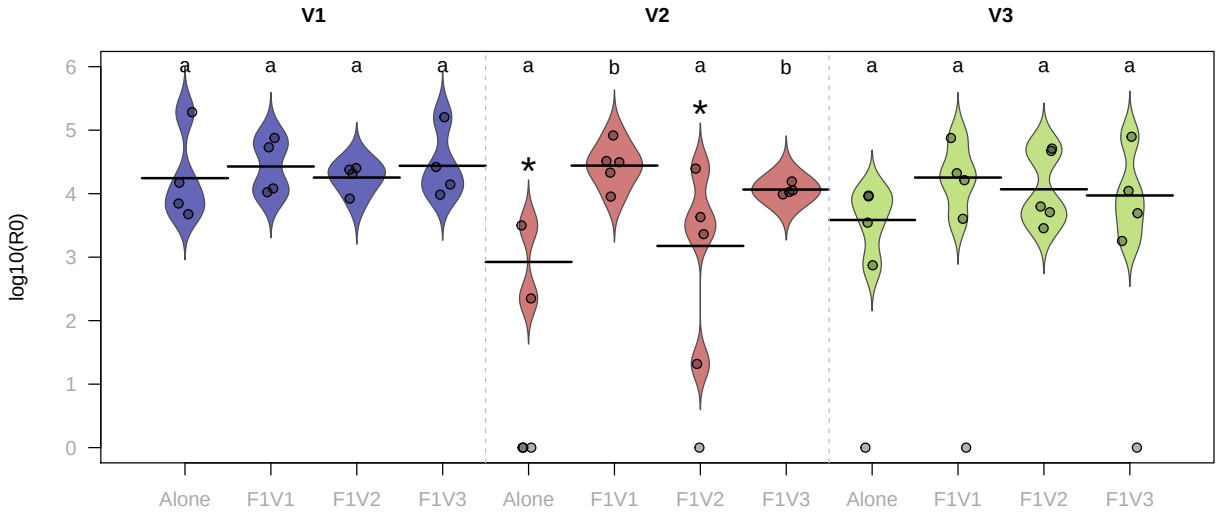

Figure 4: Transmission R_0 of GFP-labelled variants, alone or in mixture with a non-GFP variant. For each group (V1, V2 or V3), 5 GFP-labelled clones have been tested. Non-GFP clones are F1V1, F1V2 and F1V3. Differences between treatments were tested using a pairwise-Wilcoxon rank sum test. Treatments that are different from others are indicated with a star.

4 Discussion

Our *in vitro* experiment shows that in most cases, group 2 variants are more competitive than group 1 and group 3. However, in limiting resource and non-agitated conditions, which is most probably the closest condition to what happens in an insect cadaver few days after insect death, competition between variants can be intransitive, which can lead to a mixture of variants. Interestingly, this coexistence is made possible in our experiments by the group 3 variants, that can invade a group 2 population, but do not resist invasion by group 1. Although the transposition of these results to *in vivo* conditions might be problematic, on-going experiments should allow us to describe competition among variants that are co-injected in an insect hosts. Nevertheless, these first results suggest that *X. nematophila* population can be heterogeneous, with mixtures of variants that

never exclude each other during infection.

Our *in vivo* experiments demonstrates that nematodes do reproduce well, and therefore do transmit *X. nematophila*, as long as group 1 or group 3 variants are present on the onset of infection. The presence of a group 2 variant then seems to be completely neutral. From this perspective, group 2 variants cannot be considered as real cheaters, as they do not reduce group 1 or group 3 transmission, which remains similar to their transmission when injected alone. However, we should keep in mind that the transmission R_0 estimated here is that of the GFP-labelled variants, and not the overall transmission of *X. nematophila*. We furthermore show that group 2 variants can be transmitted by nematodes, even when mixed with group 1 variants. This is in agreement with former results of (Sicard et al. 2005) who showed that group 2 variants could even be better transmitted than group 1 variants in situations of competition.

Overall, our work suggests that group 2 variants, i.e. *lrp* mutants, could be maintained inside *X. nematophila* population during infection and be transmitted by nematodes. This suggests that the strong specificity of the re-association between *X. nematophila* and *S. carpocapsae*, which is well described, is not sufficient to counter select *lrp* mutants. The data we gathered so far suggest that selection could only operate when an infection is initiated by nematodes that only carry *lrp* mutants. In this case, the bad reproduction of nematodes should prevent the transmission of *lrp* mutants. Whether or not this selection is effective requires further experiments that are beyond the scope of this paper.

References

Chapuis, E., A. Arnal, and J.-B. Ferdy (2012). "Trade-offs shape the evolution of the vector-borne insect pathogen *Xenorhabdus nematophila*". *P. Roy. Soc. B-Biol. Sci.* 279.1738, pp. 2672–2680.

Cowles, K. N. et al. (2007). "The global regulator Lrp contributes to mutualism, pathogenesis and phenotypic variation in the bacterium *Xenorhabdus nematophila*". *Cell. Microbiol.* 9.5, pp. 1311–1323.

Didelot, X. et al. (2016). "Within-host evolution of bacterial pathogens". *Nat. Rev. Microbiol.* 14.3, p. 150.

Finkel, S. E. (2006). "Long-term survival during stationary phase: evolution and the GASP phenotype". *Nat. Rev. Microbiol.* 4.2, p. 113.

Kerr, B. et al. (2002). "Local dispersal promotes biodiversity in a real-life game of rock–paper–scissors". *Nature* 418.6894, p. 171.

Sicard, M. et al. (2004). "Stages of infection during the tripartite interaction between *Xenorhabdus nematophila*, its nematode vector, and insect hosts". *Appl. Environ. Microbiol.* 70.11, pp. 6473–6480.

Sicard, M. et al. (2005). "Effect of phenotypic variation in *Xenorhabdus nematophila* on its mutualistic relationship with the entomopathogenic nematode *Steinernema carpocapsae*". *Parasitology* 131, pp. 687–694.

Interlude

The production of phenotypic heterogeneity during insect infection by *Xenorhabdus nematophila* is mostly due to within-host evolution, which allows the selection of GASP (Growth Advantage in Stationary Phase) variants. These GASP variants are able to resist harsh conditions of aged cadavers, but do not seem to be beneficial for transmission, as they impair nematodes reproduction. So far, their is no sign that producing heterogeneity is adaptive for *X. nematophila* (i.e. has been selected because it increased fitness). However, these GASP variants can re-associate with nematodes, and be well transmitted when they are in mixture with non-GASP variants in the insect. As GASP variants arise during infection from a non-GASP population, these mixture conditions probably represent the majority of the cases in nature. Thus, we could expect to find high proportions of GASP variants associated to nematodes when sampling natural populations. Yet, freshly sampled nematodes have never been reported to carry GASP variants so far.

Several hypothesis could explain this paradox, and one of them is that GASP variants lack the production of exo-enzymes, such as antimicrobial compounds. Indeed, our experiments were performed in lab condition, using reared insects, which makes a pretty "clean" environment for *Xenorhabdus* and *Steinernema* to complete their life cycle. In natural populations however, *Xenorhabdus* and *Steinernema* multiply for around ten days inside an insect cadaver that stays in the soil. During this time, multiple interactions with opportunistic microbes from the soil, but also other microbes that were present in the insect and in the nematode before infection can occur. GASP variants which have a reduced production of antimicrobial compounds could thus suffer from these interactions, and be counter selected in natural conditions.

This heterogeneity in the biotic environment of *Xenorhabdus*, and in particular interactions with its host and vector microbiota, need to be taken into account to understand all the implications of phenotypic heterogeneity during infection. However, these interactions have rarely been studied. The second chapter of this thesis brings some knowledge about the kind of microbial populations that can be found inside insect cadavers in a system that is closer to natural condition. It also gives a starting point to further investigate the dynamic of the bacterial populations including *Xenorhabdus* during the course of infections.

2 Microbial communities during *Xenorhabdus nematophila* infections

Since the development of Next Generation Sequencing (NGS) methods, the number of studies on microbiota have exploded. This microbiota, i.e. the cohort of microorganisms associated with a host, have been shown to play many roles in plants and animal biology (Bordenstein et al. 2015). Insects in particular, rely on their gut microbiota for diverse functions, including development and immunity, nutrition, the modulation of immune responses, gut homeostasis or protection from pathogens and toxins (see for example Engel et al. 2016; Shi et al. 2013; Broderick et al. 2014; Welte et al. 2016; Shao et al. 2017; Caccia et al. 2016).

Although *Xenorhabdus* life cycle have been extensively studied during the past decades, very little is known about its potential interactions with its vector and host microbiota. The *Steinernema-Xenorhabdus* associations have the particularity to rapidly kill the host, and spend a long time (usually around 10 days) reproducing in the host cadaver. For now, we have nearly no idea about what happens in term of microbial population inside the host, before and after insect death.

In the present work we aimed at drawing a picture of the bacterial community present inside insect cadavers after infection by *Xenorhabdus* and its vector. To do so, we first needed to choose which insect host to use. Most of *Xenorhabdus* studies are performed on *Galleria mellonella*, a hive parasite, which has the advantage of being very easy to rear, and highly susceptible to *Steinernema-Xenorhabdus* infections. However, *Steinernema-Xenorhabdus* pairs naturally infect soil-dwelling insects. The gut microbiota of lab-reared *G. mellonella* is therefore irrelevant for this study. On the other hand, sampling insects larvae from the field can be challenging. First, a proper sampling effort can be very time consuming. Second, obtaining a large number of larvae from the same species, with comparable developmental stages and with similar genetic background needs precise taxonomic skills. To circumvent these difficulties, we decided to first develop a procedure to modify the gut microbiota of reared *Tenebrio molitor* larvae by acclimating the larvae in soil samples. This procedure allowed to mimic the gut microbiota of soil-dwelling insects

while developmental stage and species were controlled. This method is described and tested in the first paper of this chapter (under submission and deposited in bioRxiv), where we asked the following questions: **Can we modify a lab-reared insect microbiota by rearing them in soil samples? Doing so, can we mimic the microbiota of soil-dwelling insects?** This validation of methodology was also the occasion of asking some questions about the stability and plasticity of microbiota composition in insects, in response to an environmental change.

We then used these soil acclimated insects to investigate the bacterial community composition within insect cadavers after infection by *Steinernema-Xenorhabdus*. *X. nematophila* is known to produce many antimicrobial molecules. For example, it produces xenorhabdicin, a phage-tail structure that kills related *Xenorhabdus* and *Photorhabdus* species (Thaler et al. 1995; Morales-Soto et al. 2011), a protein bacteriocin called xenocin, that kills a large spectrum of bacteria isolated from insects microbiota (Singh et al. 2008), as well as broad spectrum antimicrobial compounds such as xenocoumacin (Park et al. 2009) and odilorhabdin (Singh et al. 2015) that mainly target Gram positive bacteria. We thus expected *X. nematophila* to dominate the bacterial community within the host cadaver. We characterized the bacterial community composition within insects cadavers after infection by *S. carpocapsae-X. nematophila* pairs. We then compared this community with bacterial community after infection by two *Steinernema sp-X. bovienii* pairs which have contrasted virulence and antibiotic production. Doing so, we tried to answer the questions: **Does *Xenorhabdus* dominates the bacterial community in its host cadaver, before its re-association with the nematode vector? Do we find different community patterns with different *Steinernema-Xenorhabdus* pairs?** The results of this study are gathered in a paper under submission.

The last part of this chapter is a project of paper, where we compared the bacterial community composition within insect cadavers after infections performed with several doses of *X. nematophila* and/or *S. carpocapsae* pairs. Increasing the dose of *X. nematophila* should in principle increase the chances that it dominates the microbial community within the host cadaver. *X. nematophila* is vectored by nematodes, which bring their own microbiota inside the host. Increasing the dose of nematodes thus increases both the dose of *X. nematophila* and that of other members of *S. carpocapsae* microbiota. We will thus assess here the following questions: **Is *X. nematophila* more likely to dominate the bacterial community inside insect cadaver when it initiated infection with higher doses? Do we find more members of the nematode microbiota when increasing nematode dose?** These results are presented in a project of paper.

Submitted paper:

Changes in rearing conditions rapidly modify gut microbiota structure in *Tenebrio molitor* larvae

Marine Cambon[1,2], Jean-Claude Ogier[2], Anne Lanois[2], Jean-Baptiste Ferdy[1] and Sophie Gaudriault[2*]

1. Laboratoire Evolution et Diversité Biologique, CNRS-Université Paul Sabatier, Toulouse, France.

2. Laboratoire Diversité, Génome et Interaction Microorganisme Insectes, INRA-Université de Montpellier, Montpellier, France.

* Corresponding author: Sophie Gaudriault, e-mail: sophie.gaudriault@univ-montp2.fr, telephone number: 0033 467 14 48 12

Running title: Gut microbiota structure in *T. molitor*

Abstract

The gut microbiota of multicellular organisms has been shown to play a key role in their host biology. In mammals, it has an invariant component, responsible for establishing a mutualistic relationship with the host. It also contains a dynamic fraction which facilitates adaptation in response to changes in the environment. These features have been well described in mammals, but little is known about microbiota stability or plasticity in insects. We assessed changes in microbiota composition and structure in a reared insect after a change in rearing conditions. We reared *Tenebrio molitor* (*Coleoptera*, *Tenebrioninae*) larvae for five days in soil samples from two river banks and analyzed their gut microbial communities by a metabarcoding technique, using the V3-V4 region of the 16S rRNA gene and the housekeeping gene *gyrB*. We found that soil-reared insects had a significantly more diverse microbiota than the control insects and that insects reared in soil from different sites had significantly different microbiota. We confirmed this trend by absolute quantification of the two mains fluctuating taxonomic groups: the *Enterobacteriaceae* family and the *Pseudomonas* genus, dominant in the soil-reared insects and in the control insects, respectively. Our results suggest the existence of a resident microbiota in *T. molitor* gut, but indicate that rearing changes can induce rapid and profound changes in the relative abundance of some of the members of this resident microbiota.

Keywords: resident microbiota, *T. molitor*, soil acclimatization, microbiota plasticity

Background

Microorganisms have repeatedly been shown to play a key role in plant and animal biology (Bordenstein and Theis 2015). If we are to understand the biology of a pluricellular organism, we must consider its microbiota, the cohort of microorganisms associated with the host. In animals, the gut microbiota is a key component, with major effects on host physiology. For example, the mammalian gut microbiota has been the object of many studies on digestive functions with health implications (Belizário and Napolitano 2015).

The composition of the mammalian gut microbiota displays both plasticity and invariant features. The core microbiota, which consists of the microorganisms common to the majority of individuals within a population, is generally defined as the most prevalent of the microbial species detected (Shetty et al. 2017). This common fraction of the microbiota plays a fundamental role in supporting the mutualistic symbiotic relationship with the host (Candela et al. 2012). For example, changes in the human core microbiota are associated with physiological perturbations, such as obesity and Crohn's disease (Turnbaugh et al. 2009; Hedin et al. 2015). However, another key feature of the mammalian gut microbiota is its plasticity, i.e. its ability to change in composition and structure. In humans, dietary changes induce a remarkable degree of variation in gut microbiota in terms of both phylogenetic and functional composition (Candela et al. 2012). These changes depend on various factors including host age, sex, genetic make-up, immune and health status (Shetty et al. 2017), but also exposure to environmental bacteria, geographic origin and climate (Candela et al. 2012). It has been suggested that this plasticity of the human gut microbiota facilitates rapid responses to environmental changes, resulting in rapid ecological adaptation (Alberdi et al. 2016).

Most studies on the gut microbiota concern mammals. However, the use of mammals, and more generally of vertebrates, in experimental approaches raises numerous practical, financial and ethical issues. Large-scale experiments require model organisms that are easy to manipulate and can be obtained in large numbers. Insects are interesting experimental models in this respect. Although their guts contain fewer microbial species than those of mammals (Engel and Moran 2013), insects also rely on their gut microbiota for diverse functions, including development, nutrition, the modulation of immune responses, gut homeostasis, protection from pathogens and toxins (Engel and Moran 2013; Shi et al. 2013; Broderick et al. 2014; Erkosar and Leulier 2014; Caccia et al. 2016; Welte et al. 2016; Shao et al. 2017; Raymann and Moran 2018). The gut microbiota of non-social insects is principally acquired from the environment through feeding (Engel and Moran 2013). Its composition depends on environmental conditions and diet in both laboratory and wild individuals (Chandler et al. 2011; Montagna et al. 2015; Staudacher et al. 2016). For example, it has been shown for some coleopteran species that microbiota changes with geographical location, environmental condition, and life stage (Huang and Zhang 2013;

Montagna et al. 2014).

One potential limit of these previous studies is that they used either insects from the wild, which cannot be controlled for many of their characteristics, or lab-reared insects, which are controlled but have a poorly diversified microbiota. Here we used laboratory-reared *T. molitor* larvae and mimicked a soil environment by rearing the larvae for five days in different soil samples. We assessed the changes in gut microbiota composition after acclimatization to soil samples and demonstrated a large shift in gut microbial structure. We showed in addition that different soil samples induced different modifications in insect microbiota, and that the observed plasticity was probably dependent on changes in the abundance of some of the resident OTUs.

Methods

Soil samples

We sampled soil from riverside land around Montpellier in the South of France (Figure 1A): on the banks of the Hérault river near Causse-De-La-Selle (N43°49.884' E003°41.222'; CDS sample) and on those of the Lez river near Montferrier-sur-Lez (N43°40.801' E003°51.835'; MTF sample). Both soils had a sand-silt-clay composition typical of riversides on chalky substrata. The sand:silt ratio was higher for MFT than for CDS. We collected three soil subsamples from each plot. These subsamples were taken at a depth of 20 cm and were separated by a distance of 10 m. They were named CDS1, CDS2, CDS3 and MTF1, MTF2, MTF3 (Figure 1B). The use of these six soil subsamples made it possible to compare the variability in microbiota composition both between and within plots. Each soil subsample was split into four portions, each of which was placed in a 1 L plastic box (Figure 1C), in which it was mixed with heat-sterilized (20 min at 121 °C) wheat bran (1:3 (v/v) ratio, as previously described in Jung et al. 2014).

Insects

Larvae were provided by Micronutris (St-Orens, France) and fed with heat-sterilized bran before the experiment. As it was not possible to determine their precise developmental stage, but we used only larvae weighing between 20 and 50 mg, which should correspond to 13th or 14th instar individuals (Huang et al. 2011).

Rearing of *T. molitor* larvae in soil samples

We maintained laboratory-reared *T. molitor* larvae for five days in sterilized wheat bran mixed with soil samples. During this period, the larvae were incubated at 15 °C in the same humidity conditions. They were then starved for 24 hours (Figure 1D) to exclude

individuals that were infected with pathogens (which would have died within this 24 hours period) and to limit the risk that the DNA we extract comes from the larval alimentary bolus. This starvation period potentially induces a stress on insect larvae, which might in turn impact their microbiota. We imposed it on all insects, so that the potential bias it creates is identical in all treatments.

Control insects were reared in the same conditions than other insects except that they were incubated in sterile wheat bran, with no soil mixed. Control insects microbiota should thus be close to what it was for all insects before the experiment.

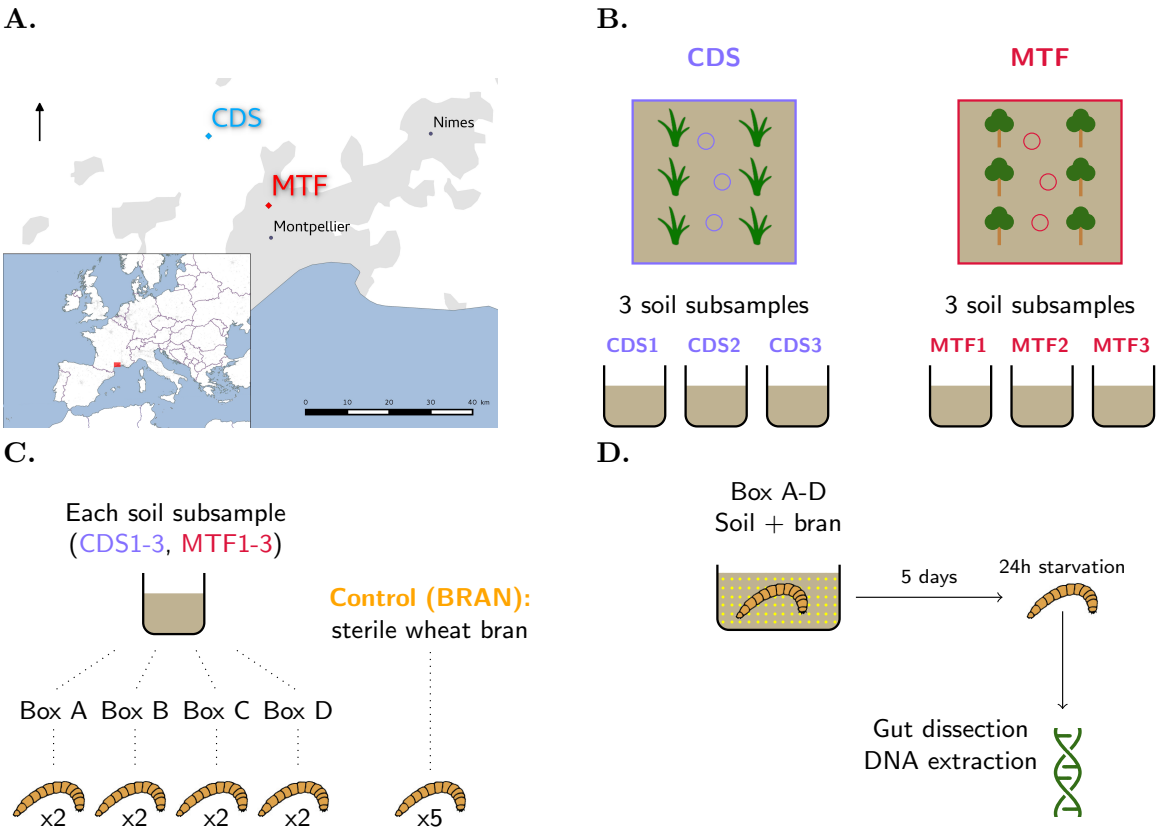

Figure 1: **Experimental design**. **A.** Location of the two sampling sites. CDS: Causse-De-La-Selle (N43°49.884' E003°41.222'; CDS sample); MTF: Montferrier-sur-Lez (N43°40.801' E003°51.835'; MTF sample). **B.** At each sampling site, we obtained three soil subsamples at positions 10 m apart. **C.** Distribution of insects in soil subsamples. Each soil subsample was split into four portions, each of which was placed in a plastic box, in which it was mixed with sterilized wheat bran. Eight insects per soil subsample (two insects/box) were analyzed. Five insects placed in a box containing sterile wheat bran only were used as a control. **D.** Insects were placed, for five days, at 15 °C, in plastic boxes containing the soil subsamples mixed with sterile wheat bran. They were then starved by incubation for 24 hours in Petri dishes. The insects were then killed, their guts were dissected, and total DNA was extracted from each gut.

DNA extraction

We extracted DNA from two randomly sampled insects per box (which makes a total of 24 insects per site) and 5 control insects. However, we failed to amplify 16S rRNA during PCR step for 2 samples, ending with 24 samples for CDS, 22 samples for MTF and 5 controls. Insect larvae were sterilized in 70 % ethanol, rinsed in water and then killed. The guts of the larvae were dissected in sterile Ringer solution (Jung et al. 2014). Dissection tools were sterilized with 70 % ethanol between insects. Dissected guts were placed in an Eppendorf tube with 100 µL of lysis solution and 1 µL lyzozyme (Quick Extract, Bacterial DNA extraction TEBU-BIO) and ground with 3 mm steel beads for 30 seconds at 20 Hz with a TissueLyzer (Qiagen). The resulting homogenates were incubated at room temperature for two days, then frozen in liquid nitrogen and heated at 95 °C to ensure that all the cells were lysed. DNA was prepared by the phenol-chloroform-alcohol and chloroform extraction method. The DNA was resuspended in sterile water and quantified with a NanoDrop spectrometer (Thermo Fisher Scientific). We performed extraction blanck controls using DNA-free water.

16S and *gyrB* DNA amplification

We targeted the V3-V4 region of the 16S rRNA gene, which is classically used for bacterial identification in microbial ecology studies, as clean and complete reference databases are available for this region. We also used the bacterial housekeeping gene *gyrB*, to support the data for the 16S rRNA (Barret et al. 2015). The V3-V4 region of the 16S rRNA gene was amplified with the PCR1F_460 (5'-ACGGRAGGCAGCAG-3') / PCR1R_460 (5'-TACCAGGGTATCTAATCCT-3') primers (modified versions of the primers used in a previous study Klindworth et al. (2012)). Amplification was performed with the MTP Taq polymerase (Sigma, ref 172-5330), according to the manufacturer's protocol, with 1 µL of 1/10 diluted DNA extract for each sample. The PCR protocol used for these primers was 60 s at 94 °C, followed by 30 cycles of 60 s at 94 °C, 60 s at 65 °C, 60 s at 72 °C, and then 10 min at 72 °C. The *gyrB* gene was amplified with primers described elsewhere: gyrB_aF64 5'-MGNCCNGSNATGTAYATHGG-3' and gyrB_aR353 5'-ACNCCRTGNARDCCDCCNGA-3' (Barret et al. 2015). Amplification was performed with the iProof High-Fidelity Taq polymerase (Bio-Rad, ref. 172-5301), according to the manufacturer's protocol, with 1 µL of 1/10 diluted DNA extract for each sample. The PCR protocol used for these primers was 30 s at 98 °C, followed by 40 cycles of 10 s at 98 °C, 30 s at 60 °C, 30 s at 72 °C, and then 10 min at 72 °C. For each PCR, we performed negative and positive controls with water and bacterial DNA extracted from a pure culture of *Xenorhabdus nematophila* (*Enterobacteriaceae*), respectively, and checked PCR amplicons by electrophoresis in a 1 % agarose gel. We performed technical replicates for the PCR and sequencing steps and obtained identical microbiota patterns (see

Additional File 2, for example). Amplicon libraries were sequenced by the GeT-Plage genomics platform at Genotoul (Toulouse, France) with Illumina MiSeq technology and a 2x250 bp kit. Raw sequence data of both 16S rRNA and *gyrB* are available from http://www.ebi.ac.uk/ena/data/view/PRJEB21797.

Metabarcoding data treatment

Sequence data for both markers were analyzed with OBITools (Boyer et al. 2015). Raw paired-end reads were aligned and merged, taking into account the phred quality score of each base to compute an alignment score. Reads with a low alignment score (>50), containing unknown bases or with an unexpected size (outside 400 bp and 470 bp, and 230 bp and 260 bp after primer trimming for the 16S rRNA gene and *gyrB* respectively) were removed from the dataset. After primer trimming, singletons (i.e. sequences only found once in the dataset) were removed (Auer et al. 2017). Sequences were then clustered into OTUs with the Sumaclust algorithm (Mercier et al. 2013), using a 97 % similarity threshold (OBITools workflows and the raw count table are available in Additional Files 3 and 4). We then removed from the datasets all clusters containing less than 0.005 % of the total number of reads (Bokulich et al. 2013). The remaining OTUs were assigned to a taxonomic group with RDPclassifier (Wang et al. 2007) and the RDPII reference database for the 16S rRNA marker and with seq_classifier.py from the mothur pipeline (Schloss et al. 2009) and the reference database from Barret et al. (2015) for *gyrB* gene (OTU assignments are available in Additional File 5).

Quantitative PCR analysis

To check for changes in OTU abundances, we performed quantitative PCR (qPCR) on two randomly picked insects per soil subsample among those used in the metabarcoding analysis. The sampling probability for each sample was adjusted for the total number of 16S rRNA reads for the sample. The five DNA samples corresponding to control insects were all analyzed.

All qPCRs were performed in triplicate, with 3 µL of reaction mixture, on a LightCycler480 machine (Roche Diagnostics), after the plate had been filled by an Echo 525 liquid handler (Labcyte). The reagent concentrations were identical in all SYBR Green I assay reactions: 1X Light-Cycler 480 SYBR Green I Master Mix (Roche Diagnostics), 500 nM each of the forward and reverse primers specific for genus *Pseudomonas* (here named *Pse*-16S, Bergmark et al. (2012)), the *Enterobacteriaceae* family (here named Entero-*rplP*, Takahashi et al. (2017)) or the *Eubacteria* kingdom (here named *uni16S*, Vandeputte et al. (2017)) (see sequences in Additional File 6) and DNA matrix. The DNA used was either genomic DNA (0.5 ng/µL) from the various reference strains, to check primer specificity (*Escherichia coli*, *Serratia marcesens*, *Klebsiella pneumoniae*, *Salmonella ty-*

phimurium, *Enterobacter cloacae*, *Pseudomonas protegens*, *Stenotrophomonas*, *Acinetobacter*, *Enterococcus*) or a 1/100 dilution of insect gut DNA for metabarcoding. The qPCR conditions were 10 minutes at 95 °C, followed by 45 cycles of 5 s at 95 °C, 10 s at 62 °C and 15 s at 72 °C, with a final dissociation curve segment. Cycle threshold (Ct) values were determined with Light-Cycler 480 software. After the validation of primer specificity ($13 < \text{Ct} < 37$ for positive controls, $\text{Ct} > 40$ for negative controls), absolute quantifications were calculated by the standard curve method. Serial dilutions of standard samples consisting of genomic DNA from *E. coli* ATCC25922 for the *rplP* gene and the rRNA16S gene (*uni16S* primers) and genomic DNA from *Pseudomonas aeruginosa* CIP76.110 (=ATCC27853) for the 16S rRNA gene (*Pse*-16S primers) were prepared and used for calibration. The gene copy number of the target gene (GCN_{target} [copies]) in standard samples was estimated using the total amount of genomic DNA in the calibration samples M_{DNA} [g], the size of the bacterial chromosome L_{DNA} [bp], the number of targets per bacterial chromosome n_{target} [copies], Avogadro's constant N_A (6.022×10^{23} bp mol^{-1}) and the mean weight of a double-stranded base pair M_{bp} (660 g mol^{-1} bp^{-1}) as follows:

$$GCN_{target} = \frac{N_A \times M_{DNA}}{L_{DNA} \times M_{bp}} \times n_{target}$$

Using the parameters of the curves linking GCN_{target} and Ct in standard samples, we then estimated the GCN of target genes in our gut samples. This estimation was possible because PCR efficiency (PE) was very close to that for standard samples (Additional File 6).

Community analysis

All analyses were performed with R version 3.3.3 (R Core Team 2015) (see Additional File 7 and 8 for the overall workflow). We did not rarefy data (McMurdie and Holmes 2014), but we used Chao1 index which is the estimated OTU richness of each sample, taking into account the possible lack of detection of some rare OTUs. Chao1 index is thus the observed OTU richness per insect plus an estimation of the unseen OTUs per insect. The Shannon index is based on relative abundance data, to represent the effective OTU richness of the sample based on the predominant OTUs. We estimated the Chao1 and Shannon alpha diversity indices with the vegan package of R (Oksanen et al. 2017). We also calculated Pielou's eveness which is the Shannon diversity divided by the natural logarithm of the OTU richness of the sample, and reflects how similar the relative abundances of OTUs in a sample are.

We calculated the beta diversity distance matrix from the Jaccard and Bray-Curtis distances for presence/absence and relative abundance data, respectively, using the vegan package. We also computed Unifrac and wUnifrac distances for presence/absence and

relative abundance data, respectively (Lozupone and Knight 2005), with the Phyloseq package (McMurdie and Holmes 2013). Unifrac and wUnifrac distances include phylogenetic distances between pairs of OTUs. A phylogenetic tree of the OTU sequences was, therefore, required. We generated this tree by aligning OTU sequences with Seaview software and the muscle method. The phylogenetic tree was built with RAxML and the GTRCAT substitution model for nucleotide sequences (Stamatakis 2014) (Additional File 9). Differences in the gut bacterial community between soil-reared insects and control insects were evaluated based on the beta diversity distance matrix, in PERMANOVA tests implemented in the vegan package (Oksanen et al. 2017), with treatment as the explanatory variable. We investigated differences between the gut bacterial communities of soil-reared insects, by performing PERMANOVA tests on distance matrices with two explanatory variables: soil sample (i.e. CDS or MTF) and soil subsample (i.e. CDS1-3, MTF1-3). Beta-diversity distances were represented using a PcoA analysis from the vegan package (Oksanen et al. 2017).

Results

Incubation of *T. molitor* larvae with soil increases the richness and diversity of their gut microbiota

After cleaning, the total dataset of the metabarcoding experiment contained 792,395 sequences clustered into 106 bacterial OTUs. Rarefaction curves showed that most of the samples had reached the saturation plateau (Figure 2A). We used the Chao1 index, which assesses the extrapolated richness of OTUs, including an estimation for undetected rare OTUs, to compare alpha diversity between soil-reared and control insects. The mean Chao1 index of the microbiota of soil-reared insects (MTF and CDS) was a 48 ± 13 OTUs whereas that of control insects (BRAN) was 25 ± 9 OTUs (Figure 2B). The OTU richness of the gut microbiota therefore increased significantly after the incubation of the insects with soil samples (Chao1 index, soil vs. control: Wilcoxon rank sum test, W=221, p-value = 1e-3). A similar conclusion was drawn for analyses based on the Shannon index, which reflects relative OTU abundance within samples (Figure 2B, soil vs. control: Wilcoxon rank sum test, W=216, p-value = 1e-3). Moreover, control insects harbored bacterial communities dominated by a very small number of dominant OTUs (low Shannon index $\simeq 0.2$ and low Pielou's eveness $\simeq 0.02$). OTU assignment identified these dominant OTUs as belonging to the *Pseudomonadaceae* family (Figure 2C). By contrast, soil-reared insects harbored bacterial communities with more balanced relative OTU abundances (Shannon index $\simeq 1.7$). The gut microbiota of these insects was dominated by *Enterobacteriaceae*, together with *Pseudomonadaceae* and other less frequent families, such as *Moraxellaceae* and *Aeromonadaceae* (Figure 2C). This was confirmed by the analysis of Pielou's eveness

index which was significantly lower in control insects than in soil-reared insects (Wilcoxon rank sum test, W=0, p-value = 7.6e-7). Thus, five days in soil significantly increased the richness of the microbiota in the gut of *T. molitor* larvae, and modified the balance of OTUs present.

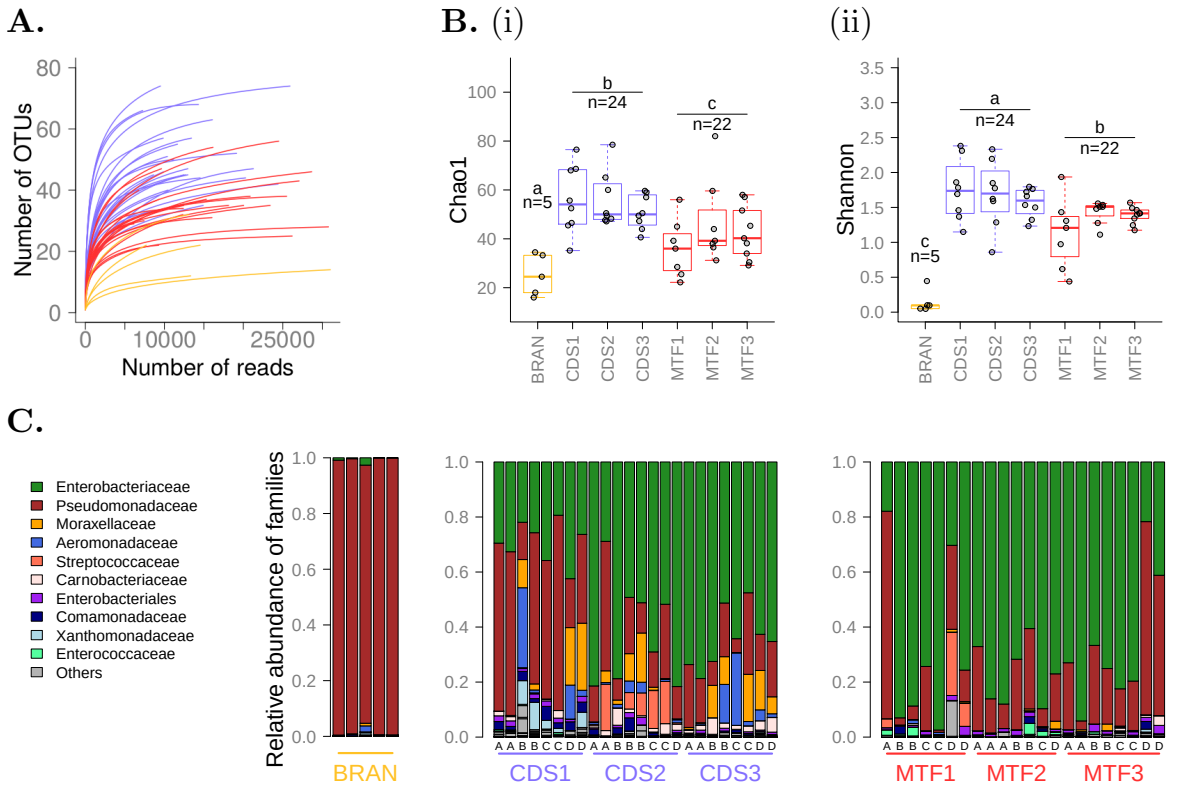

Figure 2: **Alpha diversity of the gut microbiota A.** Rarefaction curves. Each curve represents one insect. Control insects, insects reared in CDS soil samples and insects reared in MTF soil samples are shown in yellow, blue and red, respectively. **B.** Alpha diversity indices for the insect gut microbiota. CDS1-3 and MTF1-3 are the subsamples from the sampling sites (three for CDS and three for MTF). BRAN is the control treatment: insects reared on sterile wheat bran. (i) Chao1 extrapolated richness. Pairwise Wilcoxon rank-sum test, CDS-MTF: p-value = 2e-3, BRAN-CDS: p-value = 2e-3, BRAN-MTF: p-value = 0.01 (ii) Shannon diversity index. Pairwise Wilcoxon rank-sum test, CDS-MTF: p-value = 1e-3, BRAN-CDS: p-value = 5e-05, BRAN-MTF: p-value = 8e-05 **C.** Taxonomic assignment of OTUs to family level. Each bar represents an insect. Each subsample (i.e. CDS1-3 and MTF1-3) was divided into four portions, each of which was placed in a separate plastic box before the experiment. For each subsample, insects sharing the same letter (A, B, C or D) were taken from the same plastic box. The 10 families with the largest relative abundances are shown in different colors, and the others are grouped together in the "Others" category.

We also investigated the effect of soil treatments according to soil origin, by comparing the alpha diversity of CDS and MFT samples. The Chao1 and Shannon indices were significantly lower in MTF than in CDS samples (Figure 2B; Chao1 index: Kruskal-Wallis test, $\chi^2 = 12.93$, p-value = 3e-4; Shannon index: Kruskal-Wallis test, $\chi^2 = 9.6136$,

p-value = 1e-3). The CDS and MTF soils had therefore different impacts on both richness and bacterial balance.

Soil treatment induces a change in microbiota composition that is variable between soil sampling sites

We investigated the effect of soil treatment on insect microbiota, by calculating the beta-diversity between insect gut microbiota with various distance indices (Figure 3). We first calculated a distance based on pairwise Jaccard and Bray-Curtis distances. These two indices are complementary, because Jaccard distance depends purely on the presence/absence of OTUs, whereas Bray-Curtis distance also takes into account the number of reads for each OTU as a proxy for their relative abundance. We performed PCoA analysis on distance matrices (Figure 3A) where control insects tended to cluster together. PERMANOVA analysis confirmed that community composition differed between soil-reared insects and control insects (13 to 19 % of the variance explained by soil treatment, Table 1A).

The microbiota profiles of insects placed in soils from the same site (i.e. CDS or MTF) or in the same soil subsample (e.g. CDS1, CDS2 or CDS3) did not cluster together perfectly. However, a second PERMANOVA model for these samples identified two explanatory factors, soil sampling site (i.e. CDS or MTF) and subsample identity (e.g. CDS1, CDS2 or CDS3), as having a significant impact on gut community composition (Table 1B). Indeed, sample site explained 14 and 8 % of the variance and soil subsample explained 17 and 18 % of the variance, for the Jaccard and Bray-Curtis indices, respectively.

As reported above, the soil-reared insects had a microbiota dominated by *Enterobacteriaceae* (Figure 2C). We thus estimated Unifrac distances, which take into account the phylogenetic distances between OTUs, and wUnifrac distances, which also take relative OTU abundance into account. With these corrections, the differences between control insects and soil-reared insects were significant only when relative OTU abundance was taken into account (Figure 3; Table 1A). Subtle but significant effects of sample site and soil subsample on community composition were also observed with the Unifrac and wUnifrac indices (Figure 3; Table 1B).

Overall, our results show that soil treatment changes the community composition of the gut microbiota and that this change is detectable despite inter-individual variability. The bacterial communities present in the gut differ both between sample sites and between soil subsamples.

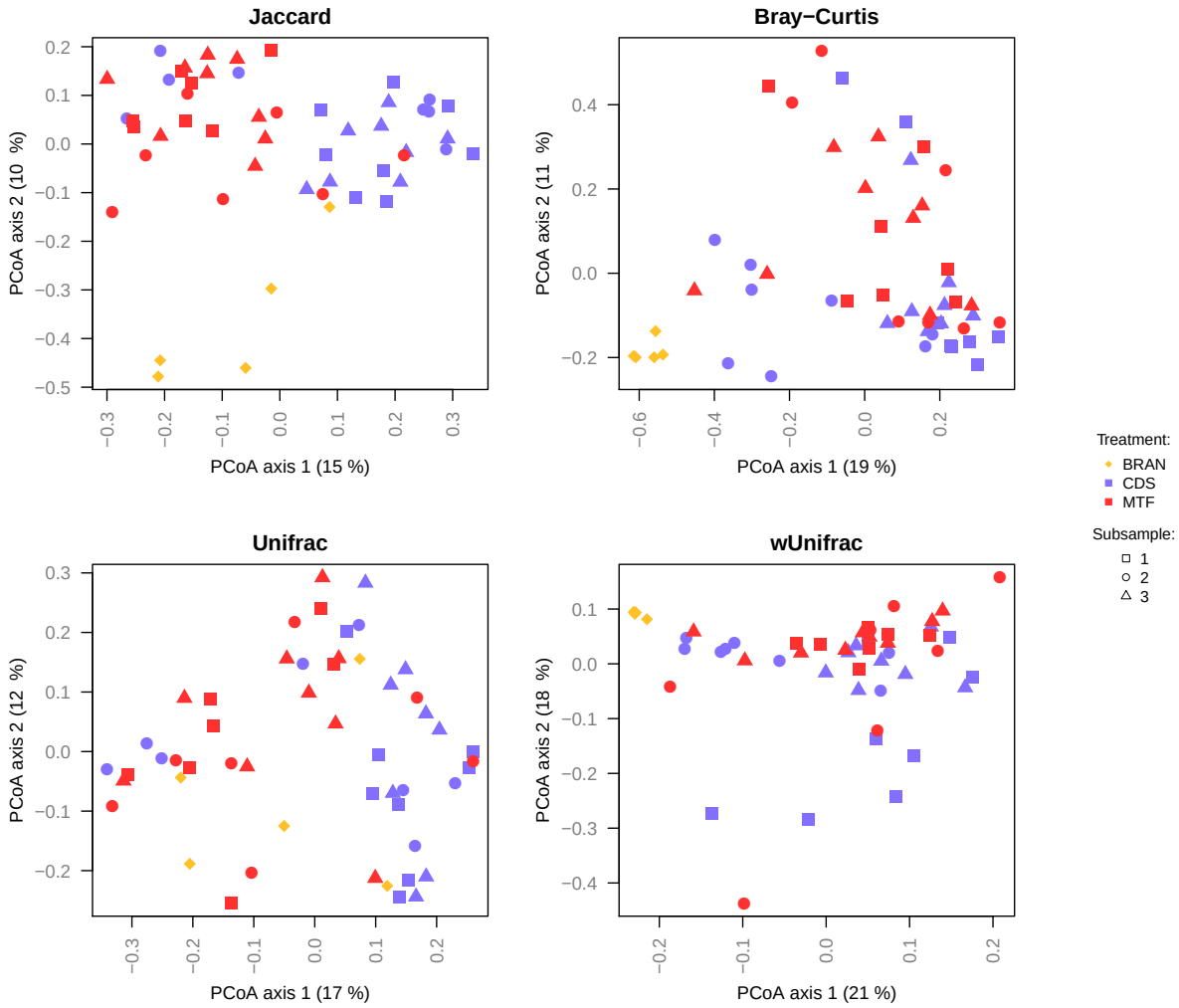

Figure 3: **PCoA analysis based on the four beta diversity distances**. Each dot corresponds to one insect. The percentage of variance explained by each axis is indicated in brackets. Yellow, blue and red dots correspond to BRAN (control), CDS and MTF samples respectively. For CDS and MTF samples, dot shape represents the identity of the soil subsample, i. e. CDS1, CDS2 and CDS3, or MTF1, MTF2 and MTF3.

Table 1: PERMANOVA analysis of the community composition of the insect microbiota based on different diversity indices, with the percentage of the variance explained by each variable and the p-value in brackets

Variable	Jacc	BC	Uni	wUni
A.	*All insects*			
Treatment	0.13 (1e-3)	0.19 (2e-3)	0.03 (0.07)	0.18 (1e-3)
B.	*Soil-reared insects*			
Site	0.14 (1e-3)	0.08 (2e-3)	0.09 (1e-3)	0.07 (6e-3)
Subsample	0.17 (1e-3)	0.18 (1e-3)	0.14 (3e-3)	0.20 (1e-3)

Jaccard distances (Jacc), Bray-Curtis distances (BC), Unifrac distances (Uni), weighted Unifrac distances (wUni). **A.** Comparison of soil-reared insects and control insects. Models contain one explanatory variable: soil treatment. **B.** Comparison of soil-reared insects. Models contained two explanatory variables: site and soil subsample

Most of the changes in the microbiota concern the relative abundances of OTUs

We then pooled all individuals of a given treatment to determine which OTUs are found in at least one individual for each treatment. The 47 OTUs found in control insects were also present in the insects of the soil treatment groups (Figure 4A). The 44 OTUs common to all three conditions matched 97 % of the reads for soil-reared insects (gray area in Figure 4B and Figure 4C). However, after soil treatment, *Pseudomonas*, the dominant OTU in control insects (98 % of the reads) accounted for only 27 and 23 % of the reads in CDS and MTF samples, respectively (Figure 4C). Conversely, *Serratia* species, together with the *Enterobacter* group, which accounted for less than 1 % of sequence reads in controls, accounted for 35 % and 43 % of the reads for CDS and MTF, respectively.

For confirmation of our initial metabarcoding results, we performed a second metabarcoding analysis with another marker, a 300 bp region of the *gyrB* housekeeping gene (see Additional File 1). This single-copy marker has been shown to provide assignments to more precise taxonomic levels than the 16S rRNA gene (Barret et al. 2015). In accordance with the results obtained with the 16S rRNA gene marker, *Pseudomonas* was the dominant OTU in control insects (more than 99 % of the reads) and its relative abundance was lower in soil-reared insects (CDS: 14 % MTF: 17 % of the reads). The genus *Serratia* and the *Enterobacter* group accounted for less than 0.06 % of the reads in control insects and a large proportion of those for the insects in the two soil treatment groups (CDS: 57 %, MTF: 70 % of the reads).

Finally, we also identified with 16S rRNA 59 OTUs that were not detectable in control insects but were present at low abundance (3 % of the reads) in at least one soil-reared

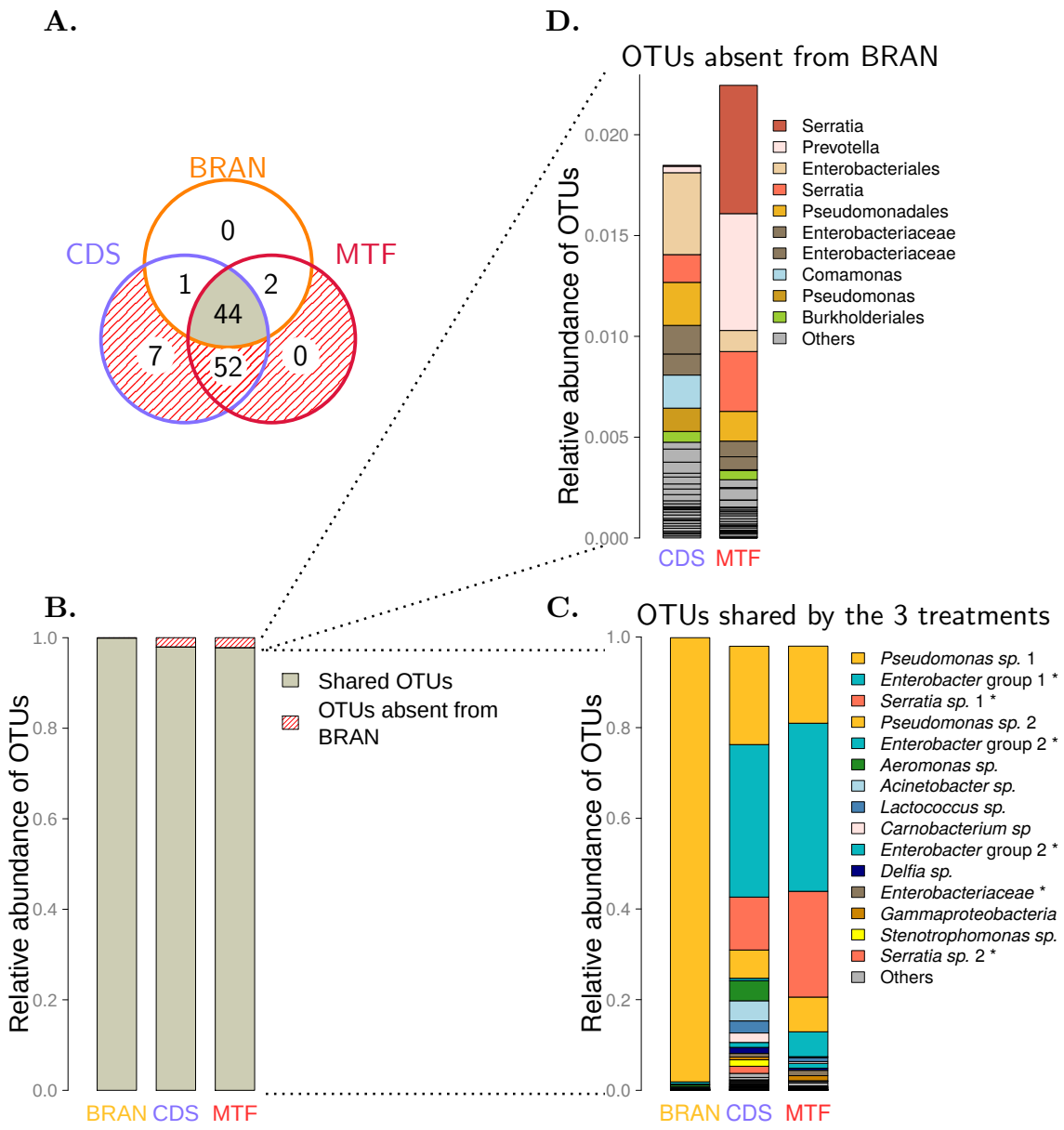

Figure 4: **Assignment of shared OTUs according to the V3-V4 region of the 16S rRNA gene A.** Venn diagram of OTUs found in at least one insect from each treatment. **B.** Bar plot of the relative abundance of the 44 OTUs common to the three treatments (in gray) and the 59 OTUs found only in soil treatments (CDS and MTF) (red stripes). The taxonomic assignment of these OTUs is detailed in **C.** and **D.**. Insects from the various treatment were pooled for these bar plots: 5 insects for BRAN, 24 insects for CDS and 22 insects for MTF. The relative abundance of OTUs was calculated from the total number of reads for each insect pool. We show here taxonomic assignments to genus level or to the lowest taxonomic level, for which the bootstrap score was < 80 %. Some OTUs differ in sequence, but were assigned to the same taxonomic group. These sequences are differentiated by a number. On each graph, the 15 OTUs with the largest relative abundance are shown in color and the others are grouped together in the "Others" category. OTU names followed by a star (*) belong to the Enterobacteriaceae family.

insect (red dashed area in Figure 4B and Figure 4D). These OTUs may correspond to taxa that were absent from the insects before soil treatment, and that colonized the insect gut during incubation in soil. Alternatively, they may have been present in the control insects at densities below the PCR detection threshold. Their abundance would then have increased above this threshold during incubation, just like the abundances of *Serratia* or *Enterobacter*. Overall, our data strongly suggest that the main effect of soil treatment is a change in the relative abundances of OTUs, although low levels of bacterial colonization from soil cannot be ruled out.

The balance between members of the resident OTUs contributes to the variation of abundances after soil treatment

We assessed the variation of OTU balance after soil treatment further, by quantifying the bacterial taxonomic groups present in all treatments but with different relative abundances between the two contrasting sets of conditions studied (control versus soil-reared). We first characterized the gut resident microbiota in our larvae, as the OTUs present in at least 95 % of our samples (following (Falony et al. 2016)). Based on 16S rRNA gene metabarcoding, we identified five resident OTUs: four *Enterobacteriaceae* (*Enterobactereriaceae* 1, *Enterobactereriaceae* 2, *Serratia* and *Enterobacter* group) and *Pseudomonas*. The resident OTUs obtained with the *gyrB* gene consisted of two OTUs, *Pseudomonas* and *Serratia*, confirming the existence of an invariant bacterial population in our insect gut microbiota. Based on the composition of this resident microbiota, we chose to monitor *Pseudomonas* and the *Enterobacteriaceae* to check for changes in the abundance of these bacteria following treatment.

We performed quantitative PCR (qPCR) on a subset of 17 samples, including the five control insects and two insects for each soil subsample. We first calculated the gene copy number (GCN) of the 16S rRNA gene in each sample, using a universal primer pair targeting *Eubacteria* (uni16S primers). As the number of 16S rRNA gene copies varies across *Eubacteria* lineages (between 1 and 15 copies per genome, Lee et al. (2008)), the GCN cannot be used to quantify the number of bacterial cells with precision (Angly et al. 2014). However, in our samples, GCN/µL ranged from 10^7 to 10^8 and did not differ significantly between samples (Kruskal-Wallis rank sum test, chi squared = 2.66, df = 2, p-value = 0.26), which suggests that the total number of bacteria was similar in our 17 samples. We then targeted a region of the 16S rRNA gene specific to the *Pseudomonas* genus, (*Pse* -16S: 251 nucleotides of the V3-V4 hypervariable region, with 4 to 7 copies per genome Bodilis et al. 2012), and a region of the *rplP* gene, region specific to the *Enterobacteriaceae* family (Entero-*rplP* : 185 nucleotides of the *rplP* gene, one copy by genome). The *Pse* -16S GCN in soil-reared insects was one tenth that in control insects (Figure 5A). Conversely, the Entero-*rplP* GCN was 100 times higher in

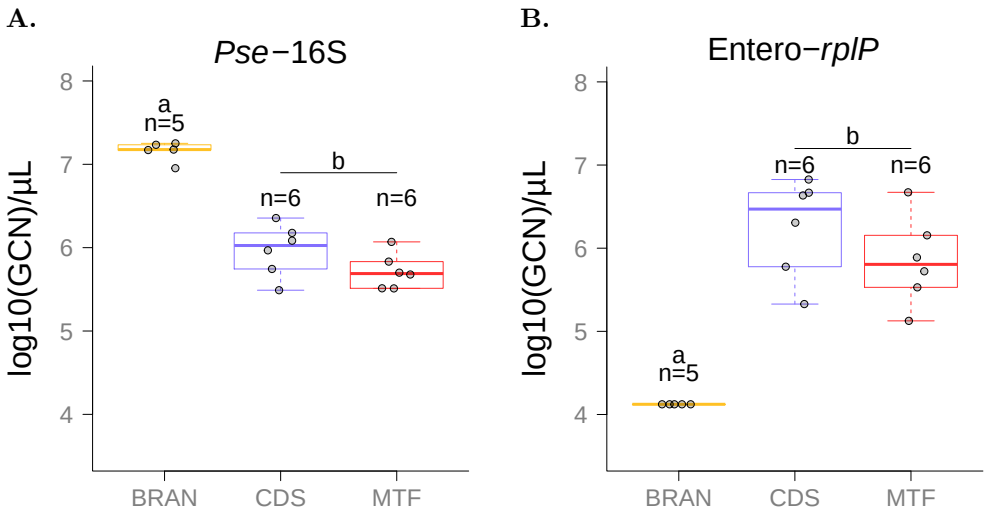

Figure 5: **Quantitative PCR on two taxa of the core microbiota A.** Gene copy number (GCN) per µL of DNA extract for Pse-16S, a specific marker of the genus Pseudomonas. Pairwise Wilcoxon rank sum test with Holm p-value adjustment, BRAN-CDS: p-value = 0.013, BRAN-MTF: p-value = 0.013, MTF-CDS: p-value = 0.18. **B.** GCN per µL of DNA extract of Entero-rplP, a specific marker of the Enterobacteriaceae family. Samples from control (BRAN) had the maximum Ct value of 40, meaning that the initial Entero-rplP quantity was under the qPCR detection threshold, i.e. < 246 GCN. Pairwise Wilcoxon rank sum test with Holm p-value adjustment, BRAN-CDS: p-value = 0.016, BRAN-MTF: p-value = 0.016, MTF-CDS: p-value = 0.31.

soil-reared insects (Figure 5B). Soil acclimation therefore seems to induce a decrease in *Pseudomonas* abundance and an increase in *Enterobacteriaceae* abundance. Our data suggest that the main effect of soil treatment is to modify the relative abundances of the resident bacterial communities of the gut microbiota.

Discussion

Rearing larvae in soil rather than in bran caused major changes in gut microbiota structure. Soil-reared larvae have a richer and more diverse gut microbiota than control larvae. Despite considerable inter-individual variability, we found that the changes in community composition depended on both the site from which the soil was obtained, and the precise soil subsample used. An analysis of the OTUs found in the different samples suggested that the main effect of the soil treatment was a change in the relative abundance of OTUs. We confirmed this trend by qPCR for the two main taxonomic groups displaying changes in abundance: the *Enterobacteriaceae* family and the genus *Pseudomonas*, which predominated in soil-reared insects and in the control, respectively.

Our rearing conditions (laboratory versus soil acclimatization) were associated with two types of gut microbial patterns, consistent with previous findings for laboratory-reared and wild insects. On the one hand, gut microbiota communities of laboratory-reared in-

sects, which are usually maintained on very simple media and diets, are dominated by one or two bacterial strains: *Pseudomonas* in our study, *Enterococcus* in moths (Chen et al. 2016; Staudacher et al. 2016) or the *Enterobacteriaceae* group *Orbus* in fruit flies (Chandler et al. 2011). On the other hand, following soil treatment, our larvae harbored more complex community profiles, with several *Enterobacteriaceae* together with the *Pseudomonas* strain that we found in control insects. Wild coleopterans, such as the forest cockchafer, *Melolontha hippocastani*, which has a soil-dwelling larval stage, have a microbiota dominated by *Enterobacteriaceae*, essentially a consortium of *Serratia*, and a Shannon diversity index close to that observed here for soil-reared insects (Arias-Cordero et al. 2012). Other coleopterans, such as *Agrilus planipennis* and *Nicrophorus vespiloides* (Vasanthakumar et al. 2008; Wang and Rozen 2017), both sampled from the wild and reared on a natural diet, also have microbiotas dominated by *Pseudomonas sp.*, the *Enterobacter* group and *Serratia sp.*. These findings suggest that our protocol can be used to mimic soil-dwelling insects effectively with reared insects. This might make it possible to obtain large numbers of individuals while working on a relevant set of bacteria in further studies of the insect gut microbiota. Moreover, we focused here on the gut microbiota, but soil treatment probably modifies the entire microbiota, including the cuticular bacterial community. Our methodology is therefore likely to be of particular interest for holobiont studies (Bordenstein and Theis 2015) involving controlled hypothesis-driven experiments on insects with a relevant total bacterial community.

The changes we observed in gut microbiota structure may result from major changes in insect diet, as insects may have access to different sources of food when incubated in soil compared to sterile bran. Our results fit well to the diet influences on microbiota documented in several *Drosophila* species (Chandler et al. 2011; Staubach et al. 2013; Vacchini et al. 2017), omnivorous cockroaches (Pérez-Cobas et al. 2015), termites (Mikaelyan et al. 2015), lepidopterans (Broderick et al. 2004; Belda et al. 2011; Priya et al. 2012) and a few coleopterans (Colman et al. 2012; Jung et al. 2014; Franzini et al. 2016; Kim et al. 2017). Changes in microbiota structure could also depend on physiochemical properties of the insect gut. In wood-feeding cockroaches, different parts of intestinal tract showed differences in pH, redox potential and hydrogen contents, and were associated to different bacterial communities (Bauer et al. 2015). The ingestion of soil particles probably modifies some of these properties of the gut. The fact that the soil characteristics differed between the two sampling sites (low sand/silt ratio for Causse-De-La-Selle (CDS), and higher sand/silt ratio for Montferrier (MTF)) could thus explain in part their different impacts on *T. molitor* gut microbiota.

The changes in the gut bacterial population may depend not only on treatment, but also on the bacterial community initially present in the gut. Previous studies (Jung et al. 2014; Osimani et al. 2018) showed that a *Spiroplasma* species predominated in the gut microbiota of the larval lineage, even after and environmental change. *Spiroplasma*

has been shown to be a heritable endosymbiont in *Drosophila* (Mateos et al. 2006). Similar effects were observed for other endosymbionts, such as *Wolbachia*, *Cardinium*, *Blattabacterium*-like and putative *Bartonella*-like symbionts in mites *Tyrophagus putrescentiae* following dietary changes (Erban et al. 2017). In all these case, endosymbiont seem to impede major shifts in the gut microbiota or conceal changes in frequencies that may occur in low-abundance OTUs. This effect is absent in our experiment, probably because the insects we used are associated to *Spiroplasma* or any other endosymbiotic bacteria.

Our results also provide interesting insight into the spatial variation of the gut bacterial community in insect populations. The differences observed after incubation in soil from different plots were consistent with the findings of other studies on coleopterans, in which the dissimilarity of the gut bacterial community between individuals is correlated with the distance between sampling sites (Adams et al. 2010). However, we also observed a difference in the gut microbiota between insects incubated with soils collected a few meters apart, at the same sampling site, and this difference was detectable despite high levels of inter-individual variation. Minor environmental differences thus have a detectable impact on the gut microbiota and structure this microbiota within insect populations over very small geographic scales.

Overall, our experiments indicate that gut microbiota can be readily changed by modifying the environment in which the insects are living. We identified resident taxa present in all the environments we tested. These taxa change in relative abundance with environmental changes. The range of environmental conditions tested here is narrower than that experienced by insects in the wild, but results suggest that, following changes in environmental conditions, the insect gut microbiota maintains a stable composition, but displays plasticity in terms of its structure.

Availability of data and material

Both the 16S rRNA and *gyrB* datasets generated and analyzed in this study are available from the ENA (European Nucleotide Archive) repository, http://www.ebi.ac.uk/ena/data/view/PRJEB21797

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Authors' contributions

MC, JBF and SG conceived the study. MC designed and performed the experiments. AL performed qPCR analysis. MC and JCO analyzed the data. JBF and SG supervised the project. All authors wrote, read and approved the final manuscript.

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References

Adams, A. S., Adams, S. M., Currie, C. R., Gillette, N. E. and Raffa, K. F. 2010. Geographic variation in bacterial communities associated with the red turpentine beetle (Coleoptera: Curculionidae). *Environmental entomology* 39, 406-414.

Alberdi, A., Aizpurua, O., Bohmann, K., Zepeda-Mendoza, M. L., and Gilbert, M. T. P. 2016. Do vertebrate gut metagenomes confer rapid ecological adaptation?. *Trends Ecol Evol* 31, 689 - 699.

Angly, F. E., Dennis, P. G., Skarshewski, A., Vanwonterghem, I., Hugenholtz, P. and Tyson, G. W. 2014. CopyRighter: a rapid tool for improving the accuracy of microbial community profiles through lineage-specific gene copy number correction. *Microbiome* 2, 11.

Arias-Cordero, E., Ping, L., Reichwald, K., Delb, H., Platzer, M. and Boland, W. 2012. Comparative evaluation of the gut microbiota associated with the below-and above-ground life stages (larvae and beetles) of the forest cockchafer, *Melolontha hippocastani*. *PLoS ONE* 7, e51557.

Auer, L., Mariadassou, M., O'Donohue, M., Klopp, C. and Hernandez-Raquet, G. 2017. Analysis of large 16S rRNA Illumina datasets: impact of singleton read filtering on microbial community description. *Mol Ecol Resour*, 00:1–11.

Barret, M., Briand, M., Bonneau, S., Préveaux, A., Valière, S., Bouchez, O., Hunault, G., Simoneau, P. and Jacques, M. A. 2015. Emergence shapes the structure of the seed microbiota. *Appl Environ Microbiol* 81, 1257-1266.

Bauer, E., Lampert, N., Mikaelyan, A., Köhler, T., Maekawa, K. and Brune, A. 2015. Physicochemical conditions, metabolites and community structure of the bacterial microbiota in the gut of wood-feeding cockroaches (Blaberidae: Panesthiinae). *FEMS Microbiol Ecol* 91, 1-14.

Belda, E., Pedrola, L., Peretó, J., Martínez-Blanch, J. F., Montagud, A., Navarro, E., Urchueguía, J., Ramón, D., Moya, A. and Porcar, M. 2011. Microbial diversity in the

midguts of field and lab-reared populations of the european corn borer *Ostrinia nubilalis*. *PLoS ONE* 6, 1-14.

Belizário, J. E. and Napolitano, M. 2015. Human microbiomes and their roles in dysbiosis, common diseases, and novel therapeutic approaches. *Front Microbiol* 6.

Bergmark, L., Poulsen, P. H. B., Al-Soud, W. A., Norman, A., Hansen, L. H. and Sørensen, S. J. 2012. Assessment of the specificity of *Burkholderia* and *Pseudomonas* qPCR assays for detection of these genera in soil using 454 pyrosequencing. *FEMS Microbiol Lett* 333, 77-84.

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., Mills, D. A. and Caporaso, J. G. 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods* 10, 57.

Bordenstein, S. R. and Theis, K. R. 2015. Host biology in light of the microbiome: ten principles of holobionts and hologenomes. *PLoS Biol* 13, e1002226.

Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P. and Coissac, E. 2015. obitools: a unix-inspired software package for DNA metabarcoding. *Mol Ecol Resour* 16, 176-182.

Broderick, N. A., Buchon, N. and Lemaitre, B. 2014. Microbiota-induced changes in *Drosophila melanogaster* host gene expression and gut morphology. *MBio* 5, e01117-14.

Broderick, N. A., Raffa, K. F., Goodman, R. M. and Handelsman, J. 2004. Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Appl and Environ Microbiol* 70, 293-300.

Caccia, S., Di Lelio, I., La Storia, A., Marinelli, A., Varricchio, P., Franzetti, E., Banyuls, N., Tettamanti, G., Casartelli, M., Giordana, B., Ferré, J., Gigliotti, S., Ecrolini, D. and Pennacchio, F. 2016. Midgut microbiota and host immunocompetence underlie *Bacillus thuringiensis* killing mechanism. *P Natl Acad Sci USA* 113, 9486-9491.

Candela, S., Biagi, E., Maccaferri, S., Turroni, S. and Brigidi, P. 2012. Intestinal microbiota is a plastic factor responding to environmental changes. *Trends Microbiol* 20, 385 - 391.

Chandler, J. A., Lang, J. M., Bhatnagar, S., Eisen, J. A. and Kopp, A. 2011. Bacterial communities of diverse *Drosophila* species: ecological context of a host-microbe model system. *PLoS Genet* 7, e1002272.

Chen, B., Teh, B. S., Sun, C., Hu, S., Lu, X., Boland, W. and Shao, Y. 2016. Biodiversity and activity of the gut microbiota across the life history of the insect herbivore *Spodoptera littoralis*. *Sci Rep* 6, 29505.

Colman, D. R., Toolson, E. C. and Takacs-Vesbach, C. D. 2012. Do diet and taxonomy influence insect gut bacterial communities?. *Mol Ecol* 21, 5124-5137.

Engel, P. and Moran, N. 2013. The gut microbiota of insects--diversity in structure and function. *FEMS Microbiol Rev* 37, 699-735.

Erban, T., Ledvinka, O., Nesvorna, M. and Hubert, J. 2017. Experimental manipulation shows a greater influence of population than dietary perturbation on the microbiome of *Tyrophagus putrescentiae*. *Appl Env Microbiol* 83, e00128-17.

Erkosar, B. and Leulier, F. 2014. Transient adult microbiota, gut homeostasis and longevity: novel insights from the *Drosophila* model. *FEBS Lett* 588, 4250-4257.

Franzini, P. Z. N., Ramond, J. B., Scholtz, C. H., Sole, C. L., Ronca, S. and Cowan, D. A. 2016. The Gut microbiomes of two *Pachysoma* Macleay desert dung beetle species (Coleoptera: Scarabaeidae: Scarabaeinae) feeding on different diets. *PLoS ONE* 11, e0161118.

Hedin, C., van der Gast, C. J., Rogers, G. B., Cuthbertson, L., McCartney, S., Stagg, A. J., Lindsay, J. O. and Whelan, K. 2015. Siblings of patients with Crohn's disease exhibit a biologically relevant dysbiosis in mucosal microbial metacommunities. *Gut*, gutjnl-2014.

Huang, Q., Hu, J., Zhou, D. G., Ling, S., Ruan, H. B., Wang, X. N., Chen, G., Zhu, T. H., Yang, C. P. and Yang, W. 2011. Comparison of growth, development, survivorship and food utilization of two color varieties of *Tenebrio molitor* (Coleoptera: Tenebrionidae). *Acta Entomol Sinica* 3, 008.

Huang, S. and Zhang, H. 2013. The impact of environmental heterogeneity and life stage on the hindgut microbiota of *Holotrichia parallela* larvae (Coleoptera: Scarabaeidae). *PloS ONE* 8, 1-14.

Jung, J., Aram, H., Park, Y. W., Kim, Y. J., Koh, H. and Woojun, P. 2014. Gut microbiota of *Tenebrio molitor* and their response to environmental change. *J Microbiol Biotechnol* 24, 888-897.

Kim, J. M., Choi, M. Y., Kim, J. W., Lee, S. A., Ahn, J. H., Song, J., Kim, S. H. and Weon, H. Y. 2017. Effects of diet type, developmental stage, and gut compartment in the gut bacterial communities of two *Cerambycidae* species (Coleoptera). *J Microbiol* 55, 21-30.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F. 2012. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res* 41, e1-e1.

Lee, Z. M. P., Bussema III, C. and Schmidt, T. M. 2008. rrn DB: documenting the number of rRNA and tRNA genes in bacteria and archaea. *Nucleic Acids Res* 37, D489-D493.

Lozupone, C. and Knight, R. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* 71, 8228-8235.

Mateos, M., Castrezana, S. J., Nankivell, B. J., Estes, A. M., Markow, T. A. and Moran, N. A. 2006. Heritable endosymbionts of *Drosophila*. *Genetics* 174, 363-376.

McMurdie, P. J. and Holmes, S. 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* 8, e61217.

McMurdie, P. J. and Holmes, S. 2014. Waste not, want not: why rarefying microbiome data is inadmissible. *PLoS Comput Biol* 10, e1003531.

Mercier, C., Boyer, F., Bonin, A. and Coissac, E. 2013. SUMATRA and SUMACLUST: fast and exact comparison and clustering of sequences.

Mikaelyan, A., Dietrich, C., Köhler, T., Poulsen, M., Sillam-Dussès, D. and Brune, A. 2015. Diet is the primary determinant of bacterial community structure in the guts of higher termites. *Mol Ecol* 24, 5284-5295.

Montagna, M., Chouaia, B., Mazza, G., Prosdocimi, E. M., Crotti, E., Mereghetti, V., Vacchini, V., Giorgi, A., De Biase, A., Longo, S., Cervo, R., Lozzia, G. C., Alma, A., Bandi, C. and Daffonchio, D. 2015. Effects of the diet on the microbiota of the Red Palm Weevil (Coleoptera: Dryophthoridae). *PLoS ONE* 10, 1-22.

Montagna, M., Gómez-Zurita, J., Giorgi, A., Epis, S., Lozzia, G. and Bandi, C. 2014. Metamicrobiomics in herbivore beetles of the genus *Cryptocephalus* (Chrysomelidae): toward the understanding of ecological determinants in insect symbiosis. *Insect Science* 22, 340-352.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, H., Szoecs, E. and Wagner, E. 2017. vegan: Community Ecology Package.

Osimani, A., Milanović, V., Cardinali, F., Garofalo, C., Clementi, F., Pasquini, M., Riolo, P., Ruschioni, S., Isidoro, N., Loreto, N., Franciosi, E., Tuohy, K., Petruzzelli, A., Foglini, M., Gabucci, C., Tonucci, F. and Aquilanti, L. 2018. The bacterial biota of laboratory-reared edible mealworms (*Tenebrio molitor* L.): From feed to frass. *Int J Food Microbiol* 272, 49 - 60.

Pérez-Cobas, A. E., Maiques, E., Angelova, A., Carrasco, P., Moya, A. and Latorre, A. 2015. Diet shapes the gut microbiota of the omnivorous cockroach *Blattella germanica*. *FEMS Microbiol Ecol* 91, fiv022.

Priya, N. G., Ojha, A., Kajla, M. K., Raj, A. and Rajagopal, R. 2012. Host plant induced variation in gut bacteria of *Helicoverpa armigera*. *PLoS ONE* 7, 1-10.

Raymann, K. and Moran, N. A. 2018. The role of the gut microbiome in health and disease of adult honey bee workers. *Curr Opin Insect Sci*.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J. and others 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 75, 7537-7541.

Shao, Y., Chen, B., Sun, C., Ishida, K., Hertweck, C. and Boland, W. 2017. Symbiont-derived antimicrobials contribute to the control of the lepidopteran gut microbiota. *Cell Chem Biol* 24, 66-75.

Shetty, S. A., Hugenholtz, F., Lahti, L., Smidt, H. and de Vos, W. M. 2017. Intestinal microbiome landscaping: insight in community assemblage and implications for microbial modulation strategies. *FEMS Microbiol Rev* 41, 182-199.

Shi, W., Xie, S., Chen, X., Sun, S., Zhou, X., Liu, L., Gao, P., Kyrpides, N. C., No, E. and Yuan, J. S. 2013. Comparative genomic analysis of the endosymbionts of herbivorous insects reveals eco-environmental adaptations: biotechnology applications. *PLoS Genet* 9, 1-12.

Stamatakis, A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312-1313.

Staubach, F., Baines, J. F., Künzel, S., Bik, E. M. and Petrov, D. A. 2013. Host species and environmental effects on bacterial communities associated with *Drosophila* in the laboratory and in the natural environment. *PLoS ONE* 8, e70749.

Staudacher, H., Kaltenpoth, M., Breeuwer, J. A. J., Menken, S. B. J., Heckel, D. G. and Groot, A. T. 2016. Variability of bacterial communities in the moth *Heliothis virescens* indicates transient association with the host. *PLoS ONE* 11, 1-21.

Takahashi, H., Saito, R., Miya, S., Tanaka, Y., Miyamura, N., Kuda, T. and Kimura, B. 2017. Development of quantitative real-time PCR for detection and enumeration of Enterobacteriaceae. *Int J of Food Microbiol* 246, 92 - 97.

R Core Team 2015. R: A Language and Environment for Statistical Computing.

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., Sogin, M. L., Jones, W. J., Roe, B. A., Affourtit, J. P. and others 2009. A core gut microbiome in obese and lean twins. *Nature* 457, 480.

Vacchini, V., Gonella, E., Crotti, E., Prosdocimi, E., Mazzetto, F., Chouaia, B., Callegari, M., Mapelli, F., Mandrioli, M., Alma, A. and Daffonchio, D. 2017. Bacterial diversity shift determined by different diets in the gut of the spotted wing fly *Drosophila suzukii* is primarily reflected on acetic acid bacteria. *Env Microbiol Rep* 9, 91-103.

Vandeputte, D., Kathagen, G., D'hoe, K., Vieira-Silva, S., Valles-Colomer, M., Sabino, J., Wang, J., Tito, R. Y., De Commer, L., Darzi, Y. and others 2017. Quantitative microbiome profiling links gut community variation to microbial load. *Nature* 551.

Vasanthakumar, A., Handelsman, J. O., Schloss, P. D., Bauer, L. S. and Raffa, K. F. 2008. Gut microbiota of an invasive subcortical beetle, *Agrilus planipennis* Fairmaire, across various life stages. *Environ Entomol* 37, 1344-1353.

Wang, Q., Garrity, G. M., Tiedje, J. M. and Cole, J. R. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73, 5261-5267.

Wang, Y. and Rozen, D. E. 2017. Gut microbiota colonization and transmission in the burying beetle *Nicrophorus vespilloides* throughout development. *Appl Environ Microbiol* 83, e03250-16.

Welte, C. U., de Graaf, R. M., van den Bosch, T. J. M., Op den Camp, H. J. M., van Dam, N. M. and Jetten, M. S. M. 2016. Plasmids from the gut microbiome of cabbage root fly larvae encode SaxA that catalyses the conversion of the plant toxin 2-phenylethyl isothiocyanate. *Environ Microbiol* 18, 1379-1390.

Additional Files

Additional files can be downloaded here: https://dl.univ-tlse3.fr/filez/n3xabn

Additional file 1: Relative abundance and taxonomic assignment of OTUs according to the *gyrB* gene

Insects from the various treatments were pooled for these bar plots: 5 insects for BRAN, 24 insects for CDS and 22 insects for MTF. The relative abundance of OTUs was calculated from the total number of reads for each insect pool. We show here taxonomic assignments to genus level or to the lowest taxonomic level for which the bootstrap score was > 80 %. Some OTUs differ in sequence but were assigned to the same taxonomic group. These sequences are differentiated by a number. On each graph, the 15 OTUs with the largest relative abundances are shown in color and the others are grouped together in the "Others" category. OTU names followed by a star (*) belong to the *Enterobacteriaceae* family.

Additional file 2: Example of a microbiota pattern in PCR replicates

We checked the reproducibility of PCR, by performing three technical PCR replicates (the three bars of the chart) on a sample chosen at random, with the whole metabarcoding procedure performed separately for each replicate. We show here the results for the CDS1D3 sample.

Additional file 3: OBITools workflow for 16S rRNA analysis

RMD_OBITools_workflow_V3V4.pdf and RMD_OBITools_workflow_gyrB.pdf contain OBITools, bash and R scripts used to obtain the OTU abundance table from raw sequencing data for both the 16S rRNA and *gyrB* genes.

Additional file 4: Raw table of reads counts

tab_div_V3V4.csv and tab_div_gyrB.csv contain raw abundance data and diversity indices for each sample, as determined with the 16S rRNA and *gyrB* genes, respectively. Samples are shown in rows and OTUs in columns.

Additional file 5: OTU taxonomic assignment

V3V4_assignment.txt is the assignment data for each 16S rRNA OTU obtained with RDPclassifier and the RDPII database. gyrB_assignment.csv is the assignment data for each *gyrB* OTU obtained with the MOTHUR classifier and the Barret et. al 2014 reference database.

Additional file 6: Primers used for qPCR

$PE_{standard}$ corresponds to PCR efficiency on gDNA standard samples, PE_{gut} corresponds to PCR efficiency on a pool of gut DNA from samples used for qPCR analysis.

Additional file 7: Statistical analysis workflow

RMD_R_workflow.pdf contains R scripts used to perform statistical analysis and to produce the figures presented in this study.

Additional file 8: R functions used in the statistical analysis workflow

- src_routine_boostrap_threshold.R is an R function for extracting the lowest taxonomic level according to a given bootstrap threshold from assignment files
- src_function_cophyloplot_modif.R is a modified version of the cophyloplot function from the ape R-package

Additional file 9: Phylogenic trees of OTUs

RAxML_bestTree_V3V4_GTRCAT and RAxML_bestTree_gyrB_GTRCAT contain the phylogenetic trees of OTUs obtained with RAxML and used for Unifrac analysis, for the 16S rRNA and gyrB genes, respectively.

Bacterial community profile after the lethal infection of *Steinernema-Xenorhabdus* pairs into *Tenebrio molitor* larvae

Marine Cambon[1,2*], Sylvie Pagès[2], Nathalie Parthuisot[1], Pierre Lafont[1], Jean-Claude Ogier[2], Jean-Baptiste Ferdy[1], Sophie Gaudriault[2*]

1. Laboratoire Evolution et Diversité Biologique, CNRS-IRD-Université Paul Sabatier, Toulouse, France.
2. Laboratoire Diversité, Génome et Interactions Microorganismes Insectes, INRA-Université de Montpellier, Montpellier, France.

*Corresponding authors: cambonmarine@gmail.com; sophie.gaudriault@umontpellier.fr

Running title: Microbiota and *Steinernema-Xenorhabdus* infection

Abstract

The host and/or vector microbiota may affect pathogens positively or negatively, altering the outcome of infections. We investigated these effects by describing the bacterial environment in which the pathogen is found before reassociation with its vector. We focused on the *Steinernema-Xenorhabdus* pairs. The entomopathogenic bacterial symbiont *Xenorhabdus* (Enterobacteriaceae) is transmitted to soil-dwelling insect larvae by infective juveniles (IJs) of the nematode *Steinernema*. We used *Tenebrio molitor* larvae that have been acclimated to soil to mimic soil-dwelling insects. We first assessed the mortality and parasitic success of three *Steinernema-Xenorhabdus* pairs. All three pairs induced lethal infections in soil-acclimated *T. molitor* larvae. We described the bacterial communities co-existing with *Xenorhabdus* at the end of insect infection, by performing V3-V4 16S rRNA metabarcoding analysis of the bacterial community within insect cadavers 10 days after infection. We found that infection modified the bacterial community, but that the level of interindividual variability remained high. Surprisingly, even for highly lethal *Steinernema-Xenorhabdus* pairs, the symbiont rarely dominated the bacterial community within the insect cadaver. Instead, these bacterial communities were generally characterized by one or two principal taxa, raising questions about the reassociation of *Xenorhabdus* with its specific vector and *Xenorhabdus* transmission.

Introduction

The recent development of sequencing techniques has made possible to study the communities of microbes associated with multicellular organisms (their microbiota) in detail. In the particular context of host-pathogen interactions, it has recently been shown that the host microbiota may have a positive or a negative impact on pathogens, potentially altering the outcome of infection (Stecher and Hardt 2008; Caccia et al. 2016; Shao et al. 2017; Onchuru et al. 2018). It is, therefore, crucial to consider the interactions between pathogens and the community of microorganisms harbored by the host during infection, to understand the factors determining the outcome of infection. Furthermore, in the specific case of vector-borne diseases, the pathogen has to deal with both the microbiota of its host, and that of its vector. Pathogen interactions with the vector microbiota and their impact on pathogen transmission are increasingly being investigated for vector-borne pathogens of mammals (Cirimotich et al. 2011; Dennison et al. 2014; Finney et al. 2015), but have been little studied for vector-borne diseases of insects.

In this study, we focused on the entomopathogenic bacterial symbiont *Xenorhabdus* (Enterobacteriaceae), which is transmitted by infective juveniles (IJs) of the nematode *Steinernema*. These IJs are soil-dwelling forms that carry small numbers of cells of the bacterium *Xenorhabdus* in an intestinal receptacle (Martens et al. 2003; Snyder et al. 2007). The IJs disperse in soil until they find a larval insect host to invade. They penetrate the insect host through natural openings, perforate the insect intestinal wall and release their *Xenorhabdus* symbionts into the hemolymph (Sicard et al. 2004; Snyder et al. 2007). The bacteria then multiply in the extracellular matrix of the host tissues, suppress the host immune system and kill the insect by septicemia (Dowds and Peters 2002; Sicard et al. 2004; Richards and Goodrich-Blair 2009). The nematodes feed on the degraded tissues and reproduce within the insect cadaver. When nutrients become limiting, the *Steinernema* IJs specifically reassociate with *Xenorhabdus* in the insect cadaver. The molecular determinants of the specific colonization of *S. carpocapsae* by *X. nematophila* have been identified (Cowles and Goodrich-Blair 2008). The symbiotic IJs then disperse into the soil in search of a new host. The interaction between *Xenorhabdus* and its vector *Steinernema* is mutualistic because the bacteria cannot survive in the soil for more than a few days in the absence of the nematode (Morgan et al. 1997), and because symbiotic *Steinernema* (*i.e.* nematodes colonized by *Xenorhabdus*) have much higher rates of reproduction than aposymbiotic nematodes (Sicard et al. 2003).

During its life cycle, *Xenorhabdus* almost certainly interacts with the microbiota of both the insect host and the nematode vector. Studies of different nematode-bacterium pairs, with host microbiota of different compositions, have shown that *Xenorhabdus* spp. dominates the bacterial community within the insect cadaver 24 hours after infection (Gouge and Snyder 2006; Singh et al. 2014), but that they coexists with other bac-

teria several days after the death of the host (Isaacson and Webster 2002; Walsh and Webster 2003). Other studies have demonstrated that, nematodes of the genus *Steinernema,* although historically considered monoxenic, can associate with bacteria other than *Xenorhabdus*, at least in laboratory (Walsh and Webster 2003; Gouge and Snyder 2006). This finding opens up new possibilities for interactions between *Xenorhabdus* spp. and other bacteria carried by the vector. However, all these studies were performed on laboratory-reared insects, which generally have a microbiota much less diverse than that of insects living in the natural environment (Chandler et al. 2011; Staudacher et al. 2016). Moreover, these studies used culture-dependent methods for the isolation and identification of bacteria, potentially resulting in an underestimation of microbiota diversity. The interactions revealed by these studies may, therefore, be far removed from what *Xenorhabdus* actually experiences in natural conditions.

In this study, we investigated the possible coexistence of *Xenorhabdus* with other bacteria present in the microbiota of the insect or the nematode vector, at the end of the infectious process. For this purpose, we reared *Tenebrio molitor* larvae in soil sampled from field, to modify the gut microbiota so that it more closely resembled that of soil-dwelling insects (Cambon et al., submitted). We then infected these insects with three *Steinernema-Xenorhabdus* pairs: *S. carpocapsae* SK27-*X. nematophila* F1, *S. feltiae* FRA44-*X. bovienii* FR44 and *S. weiseri* 583-*X. bovienii* CS03. These symbiotic pairs differ in term of host mortality and reproductive success on model insects (Sicard et al. 2004; Bisch et al. 2015). We sequenced the V3V4 region of the 16S rRNA gene, to analyze the composition of the bacterial community within the insect cadavers 10 days after infection, the timepoint at which the bacteria ressociate with their vector. We obtained strong evidence that, even for highly lethal *Steinernema-Xenorhabdus* pairs, the symbiont does not dominate the bacterial community within the insect cadavers 10 days after infection. These finding raise questions about the reassociation of *Xenorhabdus* with its specific vector, with potential implications for *Xenorhabdus* transmission.

Results

The three nematode-bacterium pairs efficiently kill both bran-reared and soil-reared *T. molitor*

We first estimated the amount of *Xenorhabdus* that the *Steinernema* strains could carry into the infested insects, by assessing the symbiont retention of the strains used for infestation: *S. carpocapsae* SK27 (Sc), *S. feltiae* FRA44 (Sf) and *S. weiseri* 583 (Sw) (Table 1). Sc carried 94±42 CFU/IJ, consistent with previous findings (Goetsch et al. 2006; Snyder et al. 2007), whereas Sf and Sw had low and very low symbiont loads (3±2 CFU/IJ and <0.05 CFU/IJ respectively).

Table 1: Insect mortality and nematodes parasitic success for the three nematode-bacterium pairs after the infestation of bran-reared *T. molitor* larvae

	Sc-Xn	**Sf-Xb**	**Sw-Xb**
Nematode strain	*S. carpocapsae* SK27	*S. feltiae* FRA44	*S. weiseri* 853
Symbiont strain	*X. nematophila* F1	*X. bovienii* FR44	*X. bovienii* CS03
Symbiont retention	94 ± 42 CFU/IJ	3 ± 2 CFU/IJ	< 0.05 CFU/IJ
Host mortality[1]	10/10	30/30	19/30
Parasitic success[2]	6/10	6/10	2/10

[1] Host mortality is the number of dead insects divided by the number of infected insects 10 days after infection, measured on bran-reared insects
[2] Parasitic success is the number of insects for which IJ emergence was observed 40 days after infection. This was measured on 10 randomly sampled dead bran-reared insects.

We then measured host mortality following the infection of *T. molitor* larvae reared in sterile wheat bran with each of the three nematode-bacterium pairs. Previous experiments revealed that these insects had a microbiota composed of only a few bacterial species, most frequently a single *Pseudomonas* strain (Cambon et al. submitted). The insects began to die two days after infestation, for all three treatments. All infected insects died within six days for the Sc-Xn and Sf-Xb treatments, whereas mortality was significantly lower (63 %, 10 days after infection) for insects infected with Sw-Xb (Table 1, binomial GLM, df=2, p-value=2.1e-05). We then followed the emergence of IJs from 10 bran-reared insect cadavers for each treatment. The IJ began to emerge about 12 days after infection, for all treatments and, 40 days after infestation, IJs had emerged from 60 % of dead insects for the Sc-Xn/Sf-Xb treatments and 20 % of dead insects for the Sw-Xb treatment (Table 1). This difference was not significant, however, probably due to the small sample size (Binomial GLM, df=2, p-value=0.104).

In a second experiment, we infected *T. molitor* larvae reared in soil samples from two different sites, CDS and MTF, so as to modify the gut microbiota to mimic that of insects from the natural environment (Figure 1A). The guts of insects reared in this way had already been shown to have a significantly more diverse microbiota than the gut of bran-reared *T. molitor* larvae (Cambon et al. submitted). All insects died after infection with Sc-Xn and Sf-Xb, in both soils (Figure 1B). For Sw-Xb, mortality reached 90 % (for larvae reared on CDS and MTF soils considered together), which is significantly higher than the 63 % of mortality recorded for bran-reared insects (Table 1 and Figure 1; binomial GLM, df=2, p-value=0.03). The difference in mortality rate between insects reared on sterile bran and those reared on soil samples suggests that bacteria from the microbiota contribute to insect death.

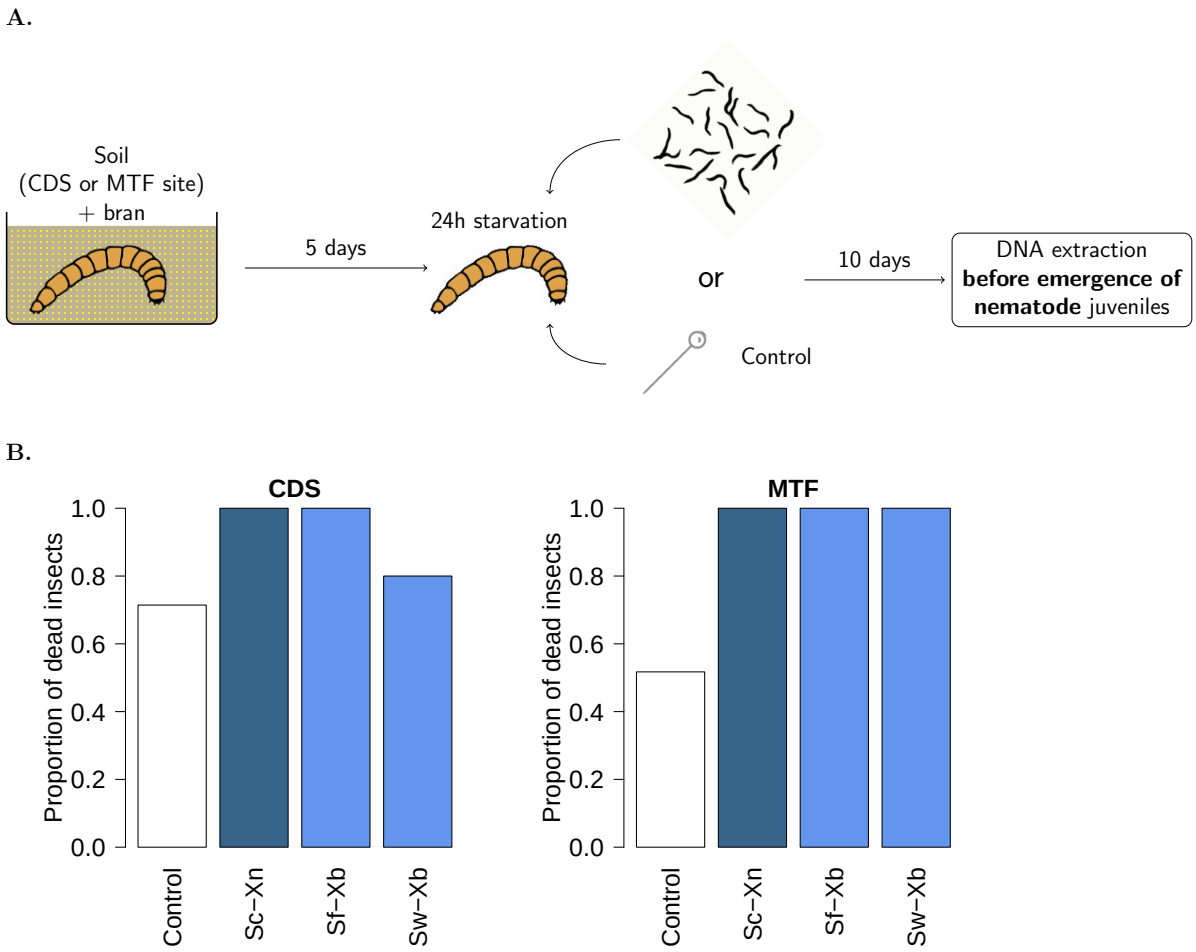


Figure 1: **Experimental design and insect mortality**. **A.** After acclimation in soil samples from the two sites, insects were infected with one of the three nematode-bacterium pairs (Sc-Xn, Sf-Xb or Sw-Xb), or were left uninfected but with wounding of their intestinal wall with a pin. After 10 days, total DNA was extracted from each insect and the V3V4 region of the 16S rRNA gene was sequenced **B.** Insects mortality 10 days after infection.

We did not infect the control insects with nematodes. Instead, their gut wall was artificially wounded with a pin through the anus to induce mortality and mimic the gut wall wound provoked by nematodes. This procedure resulted in an insect mortality of 70 % in CDS soil and 50 % in MTF soil. Mortality rates in control insect did not differ significantly between sites (Binomial GLM, df=1, p-value=0.12).

Nematode infection has a small impact on the composition and structure of the bacterial community

We sequenced the 16S rRNA V3V4 region to describe the bacterial communities found in dead soil-reared insects. After cleaning, the total dataset contained 3,827,255 sequences, clustered into 179 OTUs. The communities found in insect cadavers contained a mean

of 48±22, 61±31, 55±27 and 57±31 OTUs for controls, and for the Sc-Xn, Sf-Xb, and Sw-Xb treatments, respectively. Accordingly, the Chao1 index was significantly lower in control insects than in insects infected with nematodes, suggesting that some of the OTUs detected were supplied by the nematodes (Figure 2A, Wilcoxon rank sum test, W = 3577.5, p-value = 0.02). However, the Shannon alpha diversity index, which reflects the number of abundant taxa, did not differ significantly between treatments (Kruskal-Wallis rank sum test, chi^2 = 1.20, df = 3, p-value = 0.75), or between the controls and treatments (Figure 2B, Wilcoxon rank sum test, W = 1608, p-value = 0.53). We obtained similar results in an analysis of Pielou's evenness index, which measures the similarity between the relative abundances of the different species of the community (four treatments: Kruskal-Wallis rank sum test, chi^2 = 4.6563, df = 3, p-value = 0.2; Control vs infected: Wilcoxon rank sum test, W = 1414, p-value = 0.1446). Overall, our results suggest that *Steinernema-Xenorhabus* nfections increase the number of low frequency OTUs provided by nematodes, without affecting the diversity of abundant OTUs within insect cadavers.

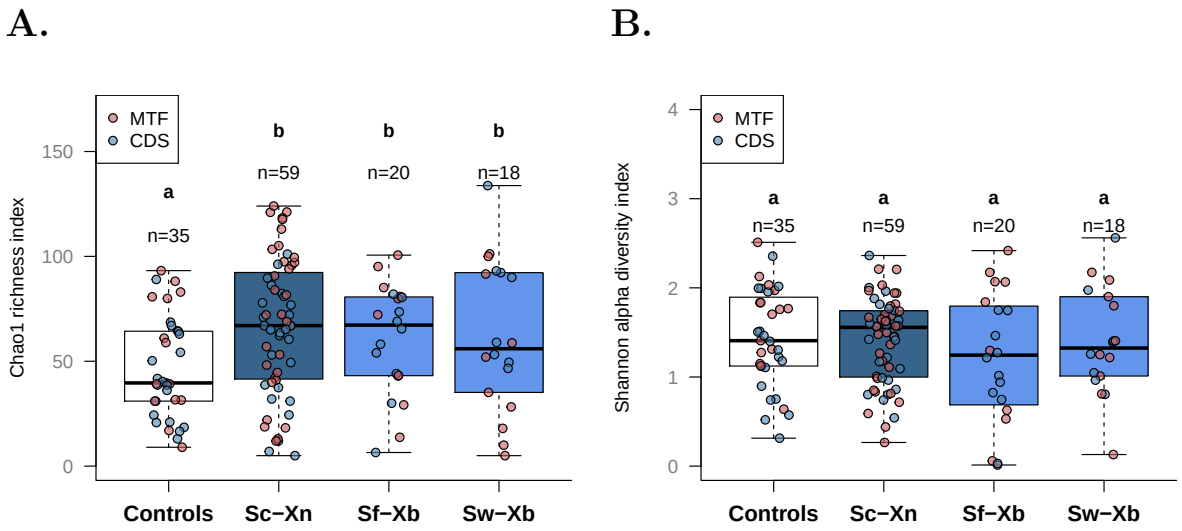


Figure 2: **Comparison of alpha diversities between treatments**. Each dot represents an insect and the color indicates the origin of the soil in which the insect was incubated. Sample sizes are given on the graph and letters indicate significant difference between groups. **A.** Chao1 richness index. **B.** Shannon alpha diversity index.

We then analyzed the beta diversity between samples (Figure 3). Surprisingly, a neighbor-joining tree based on Bray-Curtis distance did not perfectly separate control from infected insects (Figure 3). Nematode infection had a significant impact on community composition, but it accounted for only 8 % of its variance between insects (Table 2A). Soil sampling site (CDS vs MTF) accounted for 4 % of the variance, and the interaction between these two variables was not significant (Table 2A).

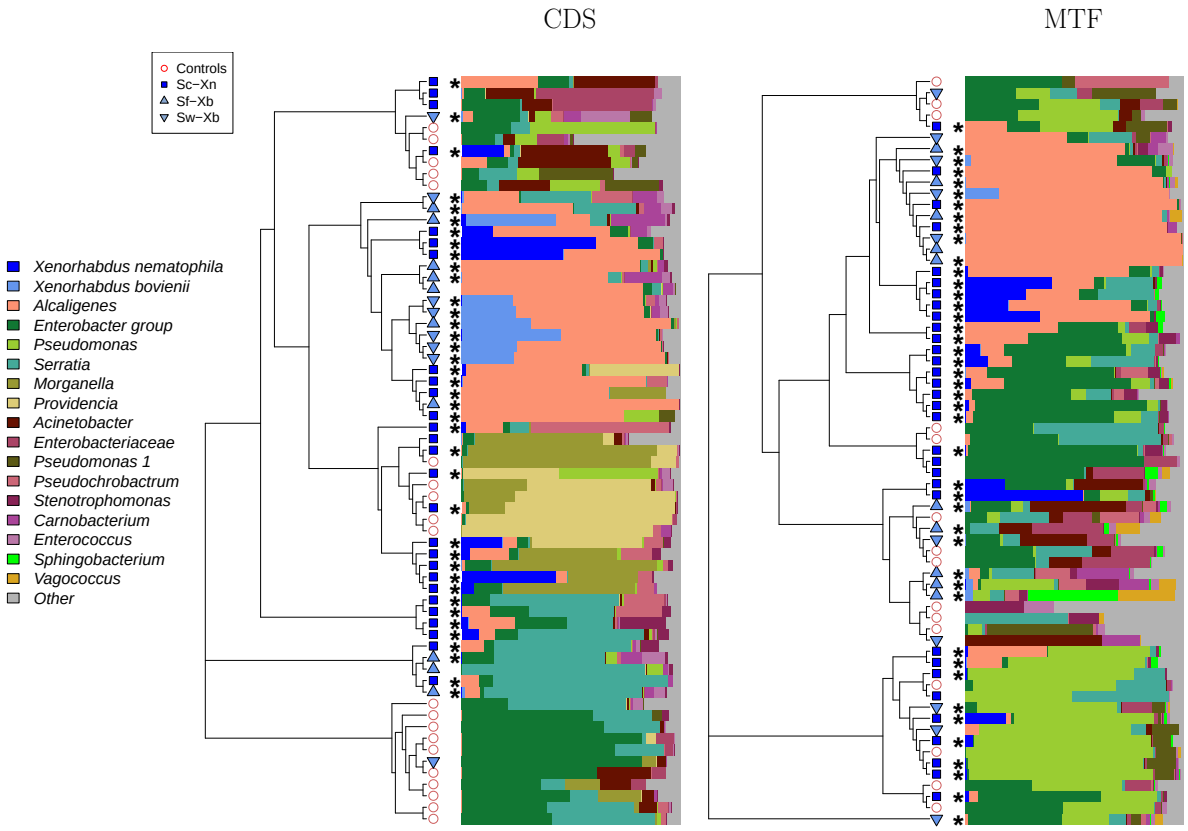


Figure 3: **Beta diversity and community composition in single insect cadavers**. Resemblance between bacteria communities is illustrated here by a neighbor-joining tree computed from a Bray-Curtis distance matrix (left, CDS; right, MTF). Stars indicate insects in which we detected
Xenorhabdus, either from metabarcoding data or by *Xenorhabdus*-specific PCR. Bars represent the relative abundance of reads matching with an OTUs in each community. On each graph, the 15 most abundant OTUs are shown in color, less frequent OTUs being grouped in the "others" category. We show here taxonomic assignments (to genus level) with booststrap scores above 80 %.

We then compared the bacterial community composition of insects infected with the three nematode-bacterium pairs. The microbiota profile of insects infected with the same nematode-bacterium pair did not strictly cluster together in Bray-Curtis distance trees (Figure3), but bacterial communities nevertheless differed significantly different between the nematode-bacterium pairs (Table 2B). However, nematode-bacterium pair accounted for only 9 % of the total variance of community composition between infected insects (Table 2B). Infection thus modified the bacterial community within insect cadavers, but considerable inter-individual variability remained.

Table 2: PERMANOVA analysis of bacterial community composition within insect cadavers

	Df	R2	p-value
A.		*all insects*[1]	
Infection	1	0.08	0.001
Site	1	0.04	0.001
Infection:Site	1	0.007	0.452
B.		*infected insects only*[2]	
Nematode-bacterium pair	2	0.09	0.001
Site	1	0.04	0.002
Pair:Site	2	0.03	0.039

[1] In the first model, all insects were taken into account. We compared control insects to infected insects (Infection), CDS sampling site to MTF sampling site (Site) and the interaction between the two explanatory variables (Infection:Site).
[2] In the second model, only infected insects were analyzed. We compared infected insects according to the nematode-bacterium pairs (Nematode-bacteria pair), the sampling sites (Site) and the interaction of these two explanatory variables (Pair:Site).

Xenorhabdus rarely invades the bacterial communities in insect cadavers

We used the he RDPII reference database for the taxonomic assignment of OTUs. Only 60 % of insect larvae infected with *Steinernema-Xenorhabdus* pairs harbored a *Xenorhabdus* OTU (Figure 3). Moreover, even in cases in which a *Xenorhabdus* OTU was detected, the relative abundance of the corresponding sample reads was generally low (Figure 3). If no *Xenorhabdus* OTU was detected by the metabarcoding approach, PCR amplifying a *Xenorhabdus*-specific region of a gene encoding an ortholog of a *tonB*-dependent heme-receptor was performed (see Material and methods). This approach made it possible to detect *Xenorhabdus* in some samples in which the relative abundance was below the metabarcoding detection threshold. *Xenorhabdus* was detected in 74 of the 97 infected insects tested (76 %; indicated by stars in Figure 3). Thus, *Xenorhabdus* was rarely the dominant genus in insect cadaver, and was even undetectable in 24 % of the infected insects.

Bacterial communities are dominated by one or two main OTUs

In 107 of the 132 insects tested (control+infected), the bacterial communities were dominated by one or two OTUs accounting for more than 40 % of the sample reads. The identity of the dominant OTU varied considerably between insects and was difficult to

predict from treatment (Table 2 and Figure 3). In 32 % of the infected insects, the OTUs were dominated by *Alcaligenes,* whereas OTUs from this genus were never dominant in control insects. We isolated an *Alcaligenes faecalis* strain from our Sc and Sw nematodes, by grinding and plating IJs. The 16S rRNA sequence of the isolated strain perfectly matched the V3V4 region sequence obtained by metabarcoding on insect cadavers. It therfore seemed likely that nematodes delivered not only *Xenorhabdus,* but also *Alcaligenes*, to the insects.

Some dominant OTUs from other species were also found in infected and control insects. These OTUs cannot therefore have come from the nematode and they probably originated instead from the insect microbiota. Some of these OTUs were found in both insects reared in MTF soil and insects reared in CDS soil. This was the case, in particular, for sequences from *Enterobacter* group bacteria and *Serratia*. Conversely, other dominant OTUs were specific to a site: *Morganella* and/or *Providencia* for CDS samples, and *Pseudomonas* for MTF samples (Figure 3). OTUs not obtained from the nematode managed to dominate the community in many insect cadavers.

Discussion

Steinernema-Xenorhabdus pairs can infect a large spectrum of insects, but they have been studied mostly on lepidopterans. Another model insect, the coleopteran *Tenebrio molitor,* had seldom been studied in this context (Solomon et al. 1999; Christen et al. 2007; Susurluk 2007; Shapiro-Ilan et al. 2008). We recently developed a procedure for acclimating *T. molitor* larvae to soil, to ensure that their gut microbiota resembles that of soil-dwelling larvae (Cambon et al, submitted). All three *Steinernema-Xenorhabdus* pairs we studied with this system were able to kill both bran-reared and soil-reared *T. molitor*. As previously shown in lepidopteran larvae (Bisch et al. 2015), the Sw-Xb pair was less pathogenic than Sc-Xn and Sf-Xb for the infection of *T. molitor* reared on sterile wheat bran. Interestingly, this difference between pathogen pairs was smaller for insects reared on soil samples: insect mortality was almost total for all pairs when soil-acclimated insects were used. This suggests that some of the bacteria present in the microbiota of soil-reared insects may opportunistically infect insects after nematodes have perforated the gut wall, contributing to insect mortality. This hypothesis is consistent with the findings of previous studies showing that infection with insect pathogens is facilitated by certain members of the host gut microbiota (Caccia et al. 2016). It is also consistent with the high mortality observed in insect controls in which the gut wall was artificially wounded with a pin through the anus (Figure 1B). We considered the host microbiota to include both the gut microbiota and the cuticular microbiota. The role of these different microbiota in the pathogenicity of nematode-bacteria pairs is beyond the scope of this study, but it would be worthwhile comparing the virulence features of *Xenorhabdus-Steinernema* pairs

infecting laboratory insects with an impoverished microbiota, and those infecting insects with a diverse, more natural, microbiota.

We used metabarcoding analysis to determine the composition of the bacterial communities found in insects killed by the three *Steinernema-Xenorhabdus* pairs. These experiments were performed on soil-reared *T. molitor* 10 days after infection, corresponding to the timepoint at which *Xenorhabdus* specifically reassociates with its vector after the sexual reproduction of nematodes and before the emergence of new IJs (Chapuis et al. 2012). *Xenorhabdus* has a genomic potential for producing numerous anti-microbial compounds (Tobias et al. 2017). We therefore expected this symbiont to dominate the bacterial community in the insect cadaver. Indeed, high densities of *Xenorhabdus* have been reported during the first few days of infection (Isaacson and Webster 2002; Walsh and Webster 2003; Singh et al. 2014). However, contrary to our expectations, we found that *Xenorhabdus* did not dominate the bacterial communities in the insect cadaver 10 days after the infection of soil-reared *T. molitor* larvae. Instead, these communities communities were mostly dominated by one or two OTUs. Some of these OTUs (*e.g. from Morganella, Providencia, Serratia, Enterobacter* species) clearly originated from the insect microbiota, as they were found in both control and infected insects. These OTUs probably took advantage of the death of the insect provoked by the nematode-bacterium pair, as previously suggested (Isaacson and Webster 2002; Walsh and Webster 2003).

The identity of the nematode-bcterium pair used for infection, despite the very different levels of *Xenorhabdus* retention of the three nematodes strains, ranging from high to very low, had also no effect on alpha diversity, community evenness and composition. Thus, despite the ability of *Xenorhabdus* to produce antibiotics, the pathogenic *Steinernema-Xenorhabdus* pair did not generally dominate the bacterial community within insect cadavers. This is particularly surprising for the Sc-Xn and Sw-Xb pairs. The xenocoumacin and odilhorhabdin produced by Xn F1 have been shown to be involved in competition with bacteria within the insect cadaver (Morales-Soto and Forst 2011; Singh et al. 2015). The genome of the Xb CS03 strain includes a number of specific loci potentially implicated in the inhibition of the microbial competitors, and Xb CS03 has antibacterial activity *in vitro* directed against species isolated from insects or nematodes (Bisch et al. 2016). The dominant OTUs from the host microbiota probably resist to the antimicrobials produdced by *Xenorhabdus,* or, alternatively, these antimicrobial compounds may no longer be produced or efficient at such late stage of infection (Isaacson and Webster 2002).

The dominant OTUs originating from the host microbiota included some from the *Enterobacter* group and others from the genus *Serratia*, after treatments with both the soil from CDS and MTF sites. Conversely, dominant OTUs from the genera *Morganella* and the *Providencia*, which probably also come from the host microbiota, were found only after treatement with CDS soil, whereas the dominant OTU from *Pseudomonas* was

found mostly after treatement with MTF soil. Thus, for a given host species, *Steinernema-Xenorhabdus* pairs may encounter and potentially interact with different sets of microorganisms according to their geographical location.

In this experimental design, *Xenorhabdus* was often found together with *Alcaligenes*, which was abundant only in infected insects and was isolated from two of our nematode strains. Our results therefore strongly suggest that *Alcaligenes*, like *Xenorhabdus*, is delivered to the insect by nematode vectors. Entomopathogenic nematodes have been shown to carry several presumably non-symbiotic bacteria that they release within infected insects. Bacteria including *Acinetobacter, Pseudomonas aureofaciens, Pseudomonas fluorescens, Enterobacter agglomerans, Serratia liquefaciens, Flavobacterium sp., Providencia vermicola, P. rettgeri, Citrobacter freundii, Staphylococcus succinus* have been isolated from various *Steinernema* strains including *S. carpocapsae* and *S. feltiae* (Walsh and Webster 2003; Gouge and Snyder 2006; Somvanshi et al. 2006; Park et al. 2011; Quiroz-Castañeda et al. 2015; Eivazian Kary 2016; Eivazian Kary et al. 2017). The specific case of *Alcaligenes faecalis* is interesting, as this bacterium has been isolated from different strains of *S. carpocapsae* and *S. feltiae* (Lysenko and Weiser 1974; Eivazian Kary et al. 2017), and we can now add *S. weiseri* to this list. *A. fecalis* is thought to be non symbiotic, but these results raise questions about its role in the life cycle of *Steinernema*. In another study, the bacterial genus *Acinetobacter* displaces the *Xenorhabdus* population to dominate bacterial community present in *G. mellonella* cadavers eight days after the start of the nematode infection (Walsh and Webster 2003). It is possible that *Alcaligenes* plays a similar role in our system.

We provide here the first evidence that IJs of three *Steinernema* species probably emerge from cadavers at a time point at which the frequency of *Xenorhabdus* is very low. Moreover, we show that the community within the host cadaver is dominated by different bacterial species that are variable according to host individuals and geographical locations. These observations probably reflect competitive interactions between *Xenorhabdus* and bacteria from the host and vector microbiota. *Xenorhabdus* may not be able to outcompete other bacteria for resource use, resulting in a lack of increase in its population density within the cadaver. The *Xenorhabdus* population may also collapse when resources become limiting, whereas other taxa, such as *Serratia*, *Pseudomonas*, and *Enterobacter* continue to increase in abundance. Our data thus raise questions about the reassociation of *Steinernema* and *Xenorhabdus* in the insect cadaver. Further studies are required to assess the impact of the host microbiota on *Xenorhabdus* transmission.

Methods

Biological material

Three nematodes species of the genus *Steinernema* were used: (i) *S. carpocapsae* SK27 associated with *X. nematophila* F1 (Sc-Xn) (Brunel et al. 1997), (ii) *S. feltiae* FRA44 associated with *X. bovienii* FR44 (Sf-Xb) (Emelianoff et al. 2008) and (iii) *S. weiseri 583* associated with *X. bovienii* CS03 (Sw-Xb) (Mrácek et al. 2003). All nematode strains had been kept for several years in the laboratory, with reamplification through the infection of *Galleria mellonella* larvae, and were stored in Ringer solution at 8 °C.

Symbiont retention, insect mortality and parasitic success measure

We assessed the retention of *Xenorhabdus* by nematodes, by surface-sterilizing 20 IJs for 10 minutes in 0.4 % bleach and rinsing them three times in sterile Ringer solution. IJs were ground with three 3 mm glass beads in 200 μL of Luria-Bertani (LB) broth for 10 minutes at 30 Hz in a TissueLyser II (Qiagen, France). The resulting suspension was plated on nutrient bromothymol blue agar (NBTA) plates (Boemare et al. 1997), on which *Xenorhabdus* form blue colonies. Blue colony-forming units (CFU) were counted to estimate the number of *Xenorhabdus* bacterial cells per nematode. Colonies with a different morphology and color from those of *Xenorhabdus* were identified by amplifiying and sequencing the 16S rRNA gene as previously described (Jiang et al. 2006).

Insect mortality and the parasitic success of nematodes were measured by infecting 30 bran-reared *T. molitor* larvae with 50 IJs in 100 μL of Ringer solution. Parasitic success was calculated as the proportion of insect cadavers displaying IJ emergence.

Rearing of insect in soil samples

We produced hosts with a microbiota as close as possible to the naturally occurring microbiota while controlling for other insect characteristics, by acclimating laboratory-reared *T. molitor* to soil samples (Cambon et al, submitted). Briefly, *T. molitor* larvae weighing between 20 and 50 mg (13th-14th instar) and provided by Micronutris (St Orens, France) were fed with heat-sterilized wheat bran before the experiment. They were then incubated for five days at 15 °C in 1 L plastic boxes containing heat-sterilized (20 min at 121°C) wheat bran (1:3 v/v) and soil (2:3 v/v). The soil used was collected from riverside sites (at Causse-De-La-Selle (CDS, N43°49.884' E003°41.222') or Montferrier-sur-Lez (MTF, N43°40.801' E003°51.835')), in three samples collected 10 meters apart and taken from a depth of 20 cm.

Insect infection with nematode-bacterium pairs

Soil-acclimated *T. molitor* larvae were placed in Eppendorf tubes (one insect per tube) on a filter paper soaked with 100 μL of Ringer solution containing 50 IJs. Control insects were not infested with nematode but their gut wall was artificially wounded with a pin through the anus, resulting in insect death. The wound created in this way mimicked the gut wall lesion caused by nematodes, and may have allowed some of the bacteria from the gut microbiota to colonize the insect general cavity in an opportunistic manner. The control insects were also placed in an Eppendorf tube on a filter paper soaked with 100 μL of Ringer solution.

All insects where then incubated at 18 °C for 10 days, corresponding to the mean time required for *Steinernema* reproduction before the emergence of the Ijs from the insect cadaver. At the end of this period, only dead insects were retained for metabarcoding analysis. We used sample size of 35, 59, 20 and 18 insects for controls, Sc-Xn, Sf-Xb and Sw-Xb respectively.

DNA extraction and V3V4 marker sequencing

Dead insect larvae were placed in an Eppendorf tube (one insect per tube) and ground with 3 mm steel beads for 30 seconds at 20 Hz with a TissueLyzer (Qiagen). 100 μL of lysis solution (Quick Extract, Bacterial DNA extraction TEBU-BIO) and 1 μL lyzozyme from the Quick Extract kit were added. Samples were incubated at room temperature for two days, frozen in liquid nitrogen and heated at 95 °C to ensure that all the cells were lysed. DNA was prepared by the phenol-chloroform-alcohol and chloroform extraction method, and precipitated in ethanol. Negative extraction controls were included in which all the extraction steps were performed in the absence of biological material, for detection of potential reagent contamination.

The V3V4 region of the 16S rRNA gene was amplified with the PCR1F_460 (5'-ACGGRAGGCAGCAG-3') and PCR1R_460 (5'-TACCAGGGTATCTAATCCT-3') primers (modified versions of the primers used in a previous study (Klindworth et al. 2012)) fused to a unique eight nucleotide tag for sample multiplexing. Some unused tag combinations were kept for the detection of potential tag switching events (see Metabarcoding data treatment). Amplification was performed with the MTP Taq polymerase (Sigma, ref 172-5330), according to the manufacturer's protocol, with 1 μL of DNA for each sample, and with the following amplification program: 60 s at 94 °C, 30 cycles of 60 s at 94 °C, 60 s at 65 °C, 60 s at 72 °C, and a final 10 minutes at 72 °C. Negative and positive controls were set up with water and bacterial DNA extracted from a pure culture of *Xenorhabdus nematophila* respectively. PCR amplicons were checked by electrophoresis in a 1 % agarose gel.

Amplicons were pooled and sequenced by the GeT-Plage genomic platform at Genotoul

(Toulouse, France) with Illumina MiSeq technology and a 2x250 bp kit. We performed technical replicates for the PCR and sequencing steps and obtained identical microbiota profiles (see Figure S1).

Metabarcoding data treatment

Sequence data were analyzed with OBITools (Boyer et al. 2015) as previously described (Cambon et al, submitted). Briefly, raw paired-end reads were aligned, merged, and alignment score was calculated. Reads with low alignment scores (<50), containing unknown bases or with an unexpected size (<400 bp or > 470 bp) were removed from the dataset. After primer trimming and sample demultiplexing, sequences found only once in the dataset were also removed (Auer et al. 2017). Sequences were then clustered into OTUs with the Sumaclust algorithm (Mercier et al. 2013), using a 97 % similarity threshold. In total, 2127 OTUs, corresponding to less than 0.005 % of the total number of reads of the dataset, were removed (Bokulich et al. 2013). The remaining OTUs were assigned to a taxonomic group with RDPclassifier (Wang et al. 2007) and the RDPII reference database.

For the removal of false-positives, we identified OTUs occurring in sequencing blank controls and calculated the total numberof these OTUs in the dataset. These OTUs were then removed from samples if their abundance was below 3 % of the total number of reads. The distribution of each OTUs in unused tag combinations was also used to filter the OTUs in each sample, by assessing the deviation of OTUs abundance values from these distribution (Esling et al. 2015).

Xenorhabdus-specific sequence amplification

If *Xenorhabdus* OTUs were not detected by metabarcoding approach in infected insects, PCR amplifying a *Xenorhabdus*-specific region was performed. A 186 bp-region of a gene, an ortholog of a *tonB*-dependent heme-receptor was targeted (XNC3v2_1960004; forward primer: ATGGCGCCAATAACCGCAACTA; reverse primer: TGGTTTCCACTTTGGTATTGATGCC) and amplified under the following conditions: 3 min at 94°C, 35 cycles of 45 sec at 94°C, 30 sec at 54°C, 30 sec at 72°C, and 10 min at 72°C. Amplification was then checked by electrophoresis in a 0.1 % agarose gel.

Community analysis

Analyses were performed with R version 3.5.0 (R Core Team 2018) as previously described (Cambon et al, submitted). Chao1, Shannon and Pielou indices and Bray-Curtis distance were estimated with the vegan package of R (Oksanen et al. 2017). Differences in alpha diversity between treatments were assessed by non parametric methods (i.e. Kruskal-

Wallis and Wilcoxon rank sum tests). The Bray-Curtis distance matrix was represented as a tree, using the neighbor-joining algorithm as implemented in the ape package (Paradis et al. 2004). Differences in bacterial community composition were assessed with PERMANOVA tests from the vegan package (Oksanen et al. 2017). A first model was adjusted on the complete dataset, with the explanatory variables infection (i.e. infected versus control insects), and site (CDS versus MTF) and the interaction between infection and site. A second model was adjusted on data for infected insects only, with the explanatory variables nematode-bacterium pair (*i.e.* Sc-Xn, Sf-Xb or Sw-Xb), and site, and the interaction between nematode-bacterium pair and site.

References

Auer, L., Mariadassou, M., O'Donohue, M., Klopp, C. and Hernandez-Raquet, G. (2017) Analysis of large 16S rRNA Illumina datasets: impact of singleton read filtering on microbial community description. Mol. Ecol. Resour. 17:e122-e132.

Bisch, G., Ogier, J.-C., Médigue, C., Rouy, Z., Vincent, S., Tailliez, P., Givaudan, A. and Gaudriault, S. (2016) Comparative genomics between two *Xenorhabdus bovienii* strains highlights differential evolutionary scenarios within an entomopathogenic bacterial species. Genome Biol. Evol. 8:148-160.

Bisch, G., Pagès, S., McMullen, J. G., Stock, S. P., Duvic, B., Givaudan, A. and Gaudriault, S. (2015) *Xenorhabdus bovienii* CS03, the bacterial symbiont of the entomopathogenic nematode *Steinernema weiseri*, is a non-virulent strain against lepidopteran insects. J. Invertebr. Pathol. 124:15-22.

Boemare, N., Thaler, J. and Lanois, A. (1997) Simple bacteriological tests for phenotypic characterization of *Xenorhabdus* and *Photorhabdus* phase variants. Symbiosis 22:167-175.

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., Mills, D. A. and Caporaso, J. G. (2013) Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat Methods 10:57.

Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P. and Coissac, E. (2015) obitools: a unix-inspired software package for DNA metabarcoding. Mol. Ecol. Resour. 16:176-182.

Brunel, B., Givaudan, A., Lanois, A., Akhurst, R. J. and Boemare, N. (1997) Fast and accurate identification of *Xenorhabdus* and *Photorhabdus* species by restriction analysis of PCR-amplified 16S rRNA genes. Appl. Environ. Microbiol. 63:574-80.

Caccia, S., Di Lelio, I., La Storia, A., Marinelli, A., Varricchio, P., Franzetti, E., Banyuls, N., Tettamanti, G., Casartelli, M., Giordana, B. and others (2016) Midgut microbiota and host immunocompetence underlie *Bacillus thuringiensis* killing mechanism.

P. Natl. Acad. Sci. USA 113:9486-9491.

Chandler, J. A., Lang, J. M., Bhatnagar, S., Eisen, J. A. and Kopp, A. (2011) Bacterial communities of diverse *Drosophila* species: ecological context of a host--microbe model system. PLOS Genet 7:e1002272.

Chapuis, E., Arnal, A. and Ferdy, J.-B. (2012) Trade-offs shape the evolution of the vector-borne insect pathogen *Xenorhabdus nematophila*. P. Roy. Soc. B-Biol. Sci. 279:2672-2680.

Christen, J., Campbell, J., Lewis, E., Shapiro-Ilan, D. and Ramaswamy, S. (2007) Responses of the entomopathogenic nematode, *Steinernema riobrave* to its insect hosts, *Galleria mellonella* and *Tenebrio molitor*. Parasitology 134:889–898.

Cirimotich, C., Ramirez, J. and Dimopoulos, G. (2011) Native microbiota shape insect vector competence for human pathogens. Cell Host & Microbe 10:307 - 310.

Cowles, C. E. and Goodrich-Blair, H. (2008) The *Xenorhabdus nematophila* nilABC genes confer the ability of *Xenorhabdus* spp. to colonize *Steinernema carpocapsae* nematodes. J. Bacteriol. 190:4121-4128.

Dennison, N. J., Jupatanakul, N. and Dimopoulos, G. (2014) The mosquito microbiota influences vector competence for human pathogens. Curr. Opin. Insect Sci. 3:6 - 13.

Dowds, B. C. and Peters, A. (2002) Virulence mechanisms. In Entomopathogenic Nematology. Gaugler, R. (eds.). CABI, pp. 79-98.

Eivazian Kary, N., Mohammadi, D. and Girling, R. (2017) New reports on dixenic associations between the symbionts of entomopathogenic nematodes, *Photorhabdus* and *Xenorhabdus*, and non-symbiotic bacteria. J. Crop Prot. 6:497-511.

Eivazian Kary N.Cand Alizadeh, Z. (2016) Non-symbiotic association of *Citrobacter freundii* and *Staphylococcus succinus* with the entomopathogenic nematode *Steinernema feltiae*. J Entomol Soc Iran:111-119.

Emelianoff, V., Le Brun, N., Pagès, S., Stock, S. P., Tailliez, P., Moulia, C. and Sicard, M. (2008) Isolation and identification of entomopathogenic nematodes and their symbiotic bacteria from Hérault and Gard (Southern France). J. Invertebr. Pathol. 98:211-217.

Esling, P., Lejzerowicz, F. and Pawlowski, J. (2015) Accurate multiplexing and filtering for high-throughput amplicon-sequencing. Nucleic Acids Res. 43:2513-2524.

Finney, C. A. M., Kamhawi, S. and Wasmuth, J. D. (2015) Does the arthropod microbiota impact the establishment of vector-borne diseases in mammalian hosts?. PLoS Pathog. 11:1-8.

Goetsch, M., Owen, H., Goldman, B. and Forst, S. (2006) Analysis of the PixA inclusion body protein of *Xenorhabdus nematophila*. J Bacteriol 188:2706-2710.

Gouge, D. H. and Snyder, J. L. (2006) Temporal association of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) and bacteria. J. Invertebr. Pathol. 91:147-157.

Isaacson, P. and Webster, J. (2002) Antimicrobial activity of *Xenorhabdus* sp. RIO

(Enterobacteriaceae), symbiont of the entomopathogenic nematode, *Steinernema riobrave* (Rhabditida: Steinernematidae). J. Invertebr. Pathol. 79:146-153.

Jiang, H., Dong, H., Zhang, G., Yu, B., Chapman, L. R. and Fields, M. W. (2006) Microbial diversity in water and sediment of Lake Chaka, an athalassohaline lake in northwestern China. Appl. Environ. Microbiol. 72:3832-3845.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F. O. (2012) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41:e1-e1.

Lysenko, O. and Weiser, J. (1974) Bacteria associated with the nematode *Neoaplectana carpocapsae* and the pathogenicity of this complex for *Galleria mellonella* larvae. J. Invertebr. Pathol. 24:332-336.

Martens, E. C., Heungens, K. and Goodrich-Blair, H. (2003) Early colonization events in the mutualistic association between *Steinernema carpocapsae* nematodes and *Xenorhabdus nematophila* bacteria. J Bacteriol 185:3147-3154.

Mercier, C., Boyer, F., Bonin, A. and Coissac, E. (2013) SUMATRA and SUMACLUST: fast and exact comparison and clustering of sequences. http://metabarcoding.org/sumatra

Morales-Soto, N. and Forst, S. A. (2011) The xnp1 P2-like tail synthesis gene cluster encodes xenorhabdicin and is required for interspecies competition. J. Bacteriol. 193:3624-3632.

Morgan, J., Kuntzelmann, V., Tavernor, S., Ousley, M. and Winstanley, C. (1997) Survival of *Xenorhabdus nematophilus* and *Photorhabdus luminescens* in water and soil. J. Appl. Microbiol. 83:665-670.

Mrácek, Z., Sturhan, D. and Reid, A. (2003) *Steinernema weiseri* n. sp.(Rhabditida, Steinernematidae), a new entomopathogenic nematode from Europe. Syst. Parasitol. 56:37-47.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E. and Wagner, H. (2017) vegan: Community Ecology Package. `https://CRAN.R-project.org/package=vegan`

Onchuru, T. O., Martinez, A. and Kaltenpoth, M. (2018) The cotton stainer's gut microbiota suppresses infection of a co-transmitted trypanosomatid parasite. Mol. Ecol. 27:3408-3419.

Paradis, E., Claude, J. and Strimmer, K. (2004) APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20:289-290.

Park, H. W., Kim, Y. O., Ha, J.-S., Youn, S. H., Kim, H. H., Bilgrami, A. L. and Shin, C. S. (2011) Effects of associated bacteria on the pathogenicity and reproduction of the insect-parasitic nematode *Rhabditis blumi* (Nematoda: Rhabditida). Can. J. Microbiol. 57:750-758.

Quiroz-Castañeda, R. E., Mendoza-Mejía, A., Obregón-Barboza, V., Martínez-Ocampo,

F., Hernández-Mendoza, A., Martínez-Garduño, F., Guillén-Solís, G., Sánchez-Rodríguez, F., Peña-Chora, G., Ortíz-Hernández, L. and others (2015) Identification of a new *Alcaligenes faecalis* strain MOR02 and assessment of its toxicity and pathogenicity to insects. Biomed Res. Int. 2015:570243.

Richards, G. R. and Goodrich-Blair, H. (2009) Masters of conquest and pillage: *Xenorhabdus nematophila* global regulators control transitions from virulence to nutrient acquisition. Cell. Microbiol. 11:1025-1033.

Shao, Y., Chen, B., Sun, C., Ishida, K., Hertweck, C. and Boland, W. (2017) Symbiont-derived antimicrobials contribute to the control of the cepidopteran gut microbiota. Cell Chem. Biol. 24:66-75.

Shapiro-Ilan, D., Rojas, M. G., Morales-Ramos, J. A., Lewis, E. E. and Tedders, W. L. (2008) Effects of host nutrition on virulence and fitness of entomopathogenic nematodes: lipid-and protein-based supplements in *Tenebrio molitor* diets. J. Nematol. 40:13.

Sicard, M., Brugirard-Ricaud, K., Pagès, S., Lanois, A., Boemare, N. E., Brehélin, M. and Givaudan, A. (2004) Stages of infection during the tripartite interaction between *Xenorhabdus nematophila*, its nematode vector, and insect hosts. Appl. Environ. Microbiol. 70:6473-6480.

Sicard, M., Le Brun, N., Pages, S., Godelle, B., Boemare, N. and Moulia, C. (2003) Effect of native *Xenorhabdus* on the fitness of their *Steinernema* hosts: contrasting types of interaction. Parasitol. Res. 91:520-524.

Singh, S., Orr, D., Divinagracia, E., McGraw, J., Dorff, K. and Forst, S. (2015) Role of secondary metabolites in establishment of the mutualistic partnership between *Xenorhabdus nematophila* and the entomopathogenic nematode *Steinernema carpocapsae*. Appl. Environ. Microbiol. 81:754-764.

Singh, S., Reese, J. M., Casanova-Torres, Á. M., Goodrich-Blair, H. and Forst, S. (2014) Microbial population dynamics in the hemolymph of Manduca sexta infected with *Xenorhabdus nematophila* and the entomopathogenic nematode *Steinernema carpocapsae*. Appl. Environ. Microbiol. 80:4277-4285.

Snyder, H., Stock, S. P., Kim, S.-K., Flores-Lara, Y. and Forst, S. (2007) New insights into the colonization and release processes of *Xenorhabdus nematophila* and the morphology and ultrastructure of the bacterial receptacle of its nematode host, *Steinernema carpocapsae*. Appl. Environ. Microbiol. 73:5338-5346.

Solomon, A., Paperna, I. and Glazer, I. (1999) Desiccation survival of the entomopathogenic nematode *Steinernema feltiae*: induction of anhydrobiosis. Nematology 1:61-68.

Somvanshi, V. S., Lang, E., Sträubler, B., Spröer, C., Schumann, P., Ganguly, S., Saxena, A. K. and Stackebrandt, E. (2006) *Providencia vermicola* sp. nov., isolated from infective juveniles of the entomopathogenic nematode *Steinernema thermophilum*. Int. J. Syst. Evol. Microbiol. 56:629-633.

Staudacher, H., Kaltenpoth, M., Breeuwer, J. A. J., Menken, S. B. J., Heckel, D. G. and Groot, A. T. (2016) Variability of bacterial communities in the moth *Heliothis virescens* indicates transient association with the host. PLoS One 11:1-21.

Stecher, B. and Hardt, W.-D. (2008) The role of microbiota in infectious disease. Trends Microbiol. 16:107 - 114.

Susurluk, A. (2007) Effectiveness of the entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema feltiae* against *Tenebrio molitor* (Yellow Mealworm) larvae in different soil types at different temperatures. Turkish J. Biol. 30:199-205.

R Core Team (2018) R: A Language and Environment for Statistical Computing. https://www.R-project.org/

Tobias, N. J., Wolff, H., Djahanschiri, B., Grundmann, F., Kronenwerth, M., Shi, Y.-M., Simonyi, S., Grün, P., Shapiro-Ilan, D., Pidot, S. J. and others (2017) Natural product diversity associated with the nematode symbionts *Photorhabdus* and *Xenorhabdus*. Nat. Microbiol. 2:1676.

Walsh, K. T. and Webster, J. M. (2003) Interaction of microbial populations in *Steinernema* (Steinernematidae, Nematoda) infected *Galleria mellonella* larvae. J. Invertebr. Pathol. 83:118-126.

Wang, Q., Garrity, G. M., Tiedje, J. M. and Cole, J. R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73:5261-5267.

Additional Files

Supplementary figure (Figure S1) can be downloaded here: `https://dl.univ-tlse3.fr/filez/hjy2s75ux`

Project of paper:

Impact of nematode and bacteria doses on bacterial community profile in insect cadavers

Marine Cambon, Nathalie Parthuisot, Pierre Lafont, Sylvie Pagès, Sophie Gaudriault, Jean-Baptiste Ferdy

1 Introduction

In a previous work (see page 83), we found that communities of microbes inside the insect cadaver were seldom dominated by *Xenorhabdus*. We demonstrated that, contrary to our expectations, bacterial communities were often dominated by bacteria from the host microbiota that opportunistically colonized the cadaver, or taxa brought by the nematodes vectors.

Again, this results are surprising as *Xenorhabdus* produces a wide variety of antimicrobial compounds which have been interpreted as tools to exclude potential competitors inside the insect cadaver (Thaler et al. 1995; Morales-Soto and Forst 2011; J. Singh and Banerjee 2008; Park et al. 2009; S. Singh et al. 2015). Our work therefore questions this view, but it also raises question about the functioning of the symbiotic association between the nematode *Steinernema* and its symbiont *Xenorhabdus*: it is indeed not clear that nematodes will easily re-associate with *Xenorhabdus* if they are born in bacterial communities where *Xenorhabdus* is in low relative abundance.

To better understand how the composition of the bacterial community may determine nematode reproduction and *Xenorhabdus* transmission, we infested soil acclimated *T. molitor* (see page 57) with different doses of both *X. nematophila* and *S. carpocapsae*. We then studied how this dose variation impacts the composition of the bacterial community, and in particular the frequency of *Xenorhabdus*. This was performed using a metabarcoding approach, using the V3-V4 region of 16S rRNA gene as a marker. We have recently started a complementary experiment where we analyze the same DNA samples using digital droplet PCR (ddPCR) techniques and a marker specific to *Xenorhabdus*, so that a more reliable quantification of *X. nematophila* can be obtained. In addition, we will use the same technique with a marker specific to *Steinernema* to quantify its biomass, and relate community composition to a proxy of the nematode number. As this experiment

is still in progress, the results presented here focus only on how the variation in doses determine community composition and frequency of *Xenorhabdus*.

2 Materials and methods

2.1 Insect infections with low and high retention strains of *S. carpocapsae*

The complete experimental procedure is detailed page 95. Briefly, *Tenebrio molitor* larvae were first acclimatized to soil sampled on two sites (CDS and MTF), by placing them in soil samples mixed with sterile wheat bran for 5 days. We previously showed that doing so enriches the gut microbiota of *T. molitor* larvae and mimics that of soil-dwelling insects.

Insects were then infected using two *S. carpocapsae* strains which differed by their *X. nematophila* symbiont retention. The low retention strain corresponds to the SK27 strain of *S. carpocapsae* associated to *X. nematophila* F1, whose symbiont retention has been reduced using the experimental procedure used to produce aposymbiotic nematodes (Sicard et al. 2003). This Low strain ultimately carried 2 ± 0.74 CFU/IJ. The high retention strain corresponds to the SK27 strain of *S. carpocapsae* associated to *X. nematophila* F1 which carried 68 ± 51 CFU/IJ (i.e. the classical levels of retention for this strain). All nematode strains had been kept for several years in the laboratory, with re-amplification through the infection of *Galleria mellonella* larvae, and were stored in Ringer solution at 8 °C. We assessed the retention of *Xenorhabdus* by nematodes, by surface-sterilizing 20 IJs for 10 minutes in 0.4 % bleach and rinsing them three times in sterile Ringer solution. IJs were ground with three 3 mm glass beads in 200 μL of Luria-Bertani (LB) broth for 10 minutes at 30 Hz in a TissueLyser II (Qiagen, France). The resulting suspension was plated on nutrient bromothymol blue agar (NBTA) plates (Boemare et al. 1997), on which *Xenorhabdus* forms blue colonies. Blue colony-forming units (CFU) were counted to estimate the number of *Xenorhabdus* bacterial cells per nematode.

Soil acclimated *T. molitor* larvae were infected in Eppendorf tubes (one insect per tube) on a filter paper soaked with 100 μL of Ringer solution containing either 50 low retention nematodes (Low50), 10 high retention nematodes (High10), or 50 high retention nematodes (High50).These three treatments allowed a gradient of the number of *X. nematophila* cells brought in the insect ($\approx$ 100, 680, and 3400 cells respectively). Control insects were not infected by nematodes but their gut wall was artificially wounded with a pin through the anus to induce mortality and mimic gut wall wound made by nematodes. All insects where then incubated at 18 °C for 10 days, which corresponds to the mean time required for *S. carpocapsae* reproduction before the emergence of the Ijs from the insect cadaver. We measured host mortality, and the total DNA of each insect was extracted. The V3-V4 region of the 16S rRNA gene was then amplified and sequenced to identify

the bacterial community present in the insect cadaver.

2.2 Digital droplet PCR on DNA extracts

Absolute quantification with ddPCR is currently performed with a marker specific to *Xenorhabdus* previsouly described (see page 97) and a marker specific to *Steinernema*, using the following primer : Steiner_F 5'-TATCAAGTCTTATCGGTGGATCACT-3' and Steiner_R 5'-GACCCTCAATTGAACATACTAACAGATA-3' developed by Jean-Claude Ogier (DGIMI lab).

3 Results

3.1 *Xenorhabdus* reads are more abundant in Low50 treatment

Ten days after infection, we counted the number of dead and alive insects for each treatments. Mortality rate increased as the *X. nematophila* dose used to initiate infection increased (Figure 1A), ranging from 60 % of mortality for wounded insects (Controls) to 100 % of mortality for the highest *X. nematophila* dose (High50). We found this difference among treatments to be statistically significant, while insects acclimatized with different soil samples had comparable mortality rate (Table 1).

We then analyzed the V3-V4 region of the 16S rRNA gene on DNA extracted from grounded insects, so that we can describe the composition of the bacterial communities that colonize insect cadavers. We first compared among treatments the number of reads assigned to the genus *Xenorhabdus* found in dead insects. We shall hereafter use this read number as a proxy for *Xenorhabus* density in the cadaver, which will be precisely measured later using ddPCR.

Interestingly, we did not detect a significant number of *Xenorhabdus* reads in 16 % of the insects which died after infection (Figure 1B and *tonB* receptor PCR data). This can be interpreted either as a sign that these insects were not killed by *X. nematophila*, or as an indication that *X. nematophila* killed the insect but decreased in density afterwards. A Zero-Inflated Negative Binomial regression which allows to analyze both cases where *X. nematophila* is positive and cases where no reads are detected, demonstrates

Table 1: Binomial Generalized Linear Model testing the effect of treatment and site on the proportion of dead insects.

	Df	p-value
treatment	3	2.45e-09
site	1	0.09
treatment:site	3	0.94

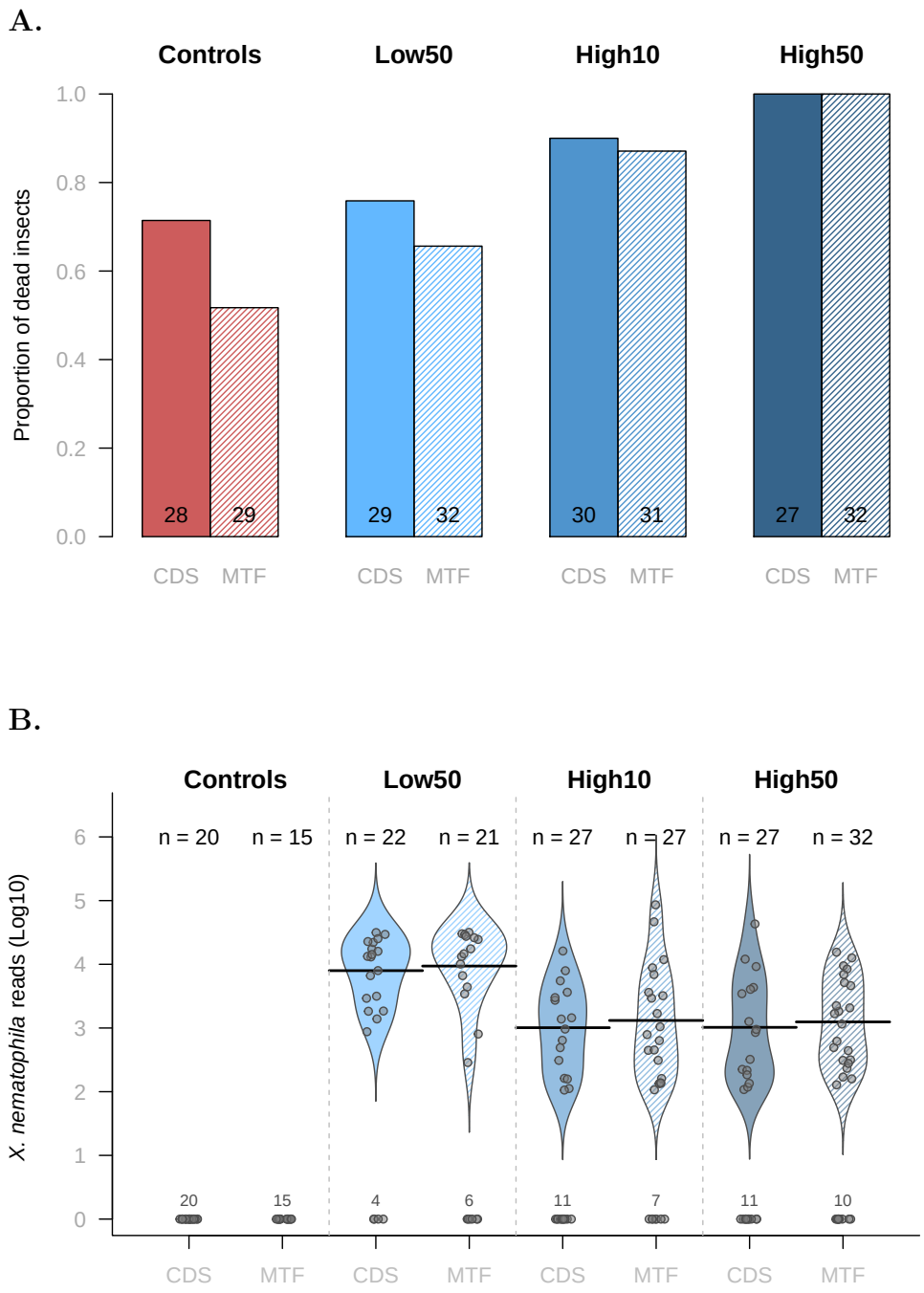


Figure 1: **Insect mortality and number of *X. nematophila* reads for each treatment**. Controls are uninfected insects artificially wounded, Low and High correspond to the retention of the nematodes strains, and 10 and 50 correspond to the number of nematodes used for the infection. **A.** Proportion of dead insects 10 days after infection. Plain bars and hatched bars are insects incubated in soil from CDS and MTF sites respectively. Mortality significantly differs between treatments but not between sites. **B.** Number of *Xenorabdus* reads found in dead insects for each treatment and each site. n is the number of dead individuals per treatment. The number of dead individuals for which no *Xenorhabdus* reads were found are indicated at the bottom of the graph

that *X. nematophila* number of reads significantly varies among treatments (Table 2). We found no significant difference in number of *X. nematophila* among sampling sites, although the interaction between the two explanatory variable was significant.

Surprisingly, a *post hoc* analysis demonstrates that *X. nematophila* reads (and its probability to be non-zero) is significantly higher in Low50 treatment compared to High10 and High50 (Table 3). The lowest frequency of zero reads in Low50 cannot be explained by the fact that insects had a higher chance to die from infection in this treatment. We indeed demonstrated that death rate is lower in Low50 than in High10 and High50. It is therefore rather an indication that the infectious process is delayed when *X. nematophila* dose is low: if correct, this interpretation would imply that insects in Low50 would have died later than insects infected with greater dose of *X. nematophila*, and that the bacterial communities we found in their cadaver are younger than those found in other insects killed by *X. nematophila*. The lower number of *X. nematophila* reads in High50 and High10 treatment would therefore be a sign that *X. nematophila* density decreases when the infection ages.

3.2 Bacterial communities in insect cadavers are dominated by few OTUs

We then analyzed the bacterial community composition for each insect cadaver. For this purpose, we calculated a distance matrix between samples, based on the Bray-Curtis beta diversity index and analyzing separately insects acclimatized with soil from different sites. The two distance matrices obtained this way can be represented as neighbour-joining trees (Figure 2). In both CDS and MTF sites, control insects (i.e. wounded but uninfected insects) cluster with some of the insects that were infected by *X. nematophila* and *S. carpocapsae*. This can mean either that the infection by *X. nematophila* failed, and that the insects died from some other reason. This can also suggests that the bacterial community of these infected insects was not influenced by the presence of nematodes-bacteria pair.

Table 2: Zero-inflated Negative Binomial regression on the number of *Xenorhabdus* reads in dead infected insects. Zero-inflated Negative Binomial regression allows to model count data, that have an excess of zero value. The number of zero is predicted separately in a binomial model, while the non-zero counts are modeled with a Negative Binomial. The two models are then combined to estimate the effect of predictive variables and their interactions, which are here the nematode treatment (excluding controls) and the site.

	Df	p-value
treatment	2	1.8e-4
site	1	0.20
treatment:site	1	0.023

Table 3: Post-hoc comparison of treatment with a zero-inflated Negative Binomial regression on the number of *Xenorhabdus* reads in dead infected insects. p-values have been adjusted here for multiple tests using the Holms method

	Low50-High10	Low50-High50	High10-High50
treat	3.5e-03	6.9e-05	1.00
site	0.11	1.00	0.35
int	0.14	0.39	0.099

The majority of infected insects, however, clustered together, meaning that some bacterial community have been shaped by the presence of the nematode-bacteria pair. However, the community remains highly variable within treatments: whether or not the insect was infected explained only 6% of the variance in community composition, while sampling site explained 3 % of the variance (Table 4A). When only infected insects are analyzed, the infection treatment (i.e. Low50, High10 and High5O) explained 7 % of the variance, and the sampling site explained 2 % (Table 4B).

Table 4: PERMANOVA analysis of bacterial community composition inside insect cadavers **A.** In the first model, all dead insects were taken into account and we compared control insects to infected insects (Infection), CDS sampling site to MTF sampling site (Site) and the interaction between the two explanatory variables (Infection:Site) **B.** In the second model, we analyzed only dead insects that were infecte by *X. nematophila* and *S. carpocapsae*. Explanatory variable are therefore treament, sampling sites and the their interaction.

	Df	R2	p-value
A.		*all insects*	
Infection	1	0.06	0.001
Site	1	0.03	0.001
Infection:Site	1	0.004	0.554
B.		*infected insects only*	
Nematode-bacteria couple	2	0.07	0.001
Site	1	0.02	0.001
Couple:Site	2	0.01	0.312

As previously found with several species of *Steinernema* and *Xenorhabdus* (see pages 83-98), the bacterial community in insect cadavers is usually dominated by one or two OTUs (Figure 2). Based on this observation, we defined the set of dominant OTUs for each sample as the minimal number of OTUs needed to reach 50 % of the reads in the sample. We found 15 OTUs that can be dominant in a sample, either alone or in mixture. The distribution of these dominant OTUs (or of these OTUs combinations) is represented on Figure 3.

These dominant OTUs can be categorized based on where we did found them:

(i) Some OTUs were probably dominant in insects microbiota before infection and still dominate the community after host death. It is the case for *Enterobacter*, *Acinetobacter*

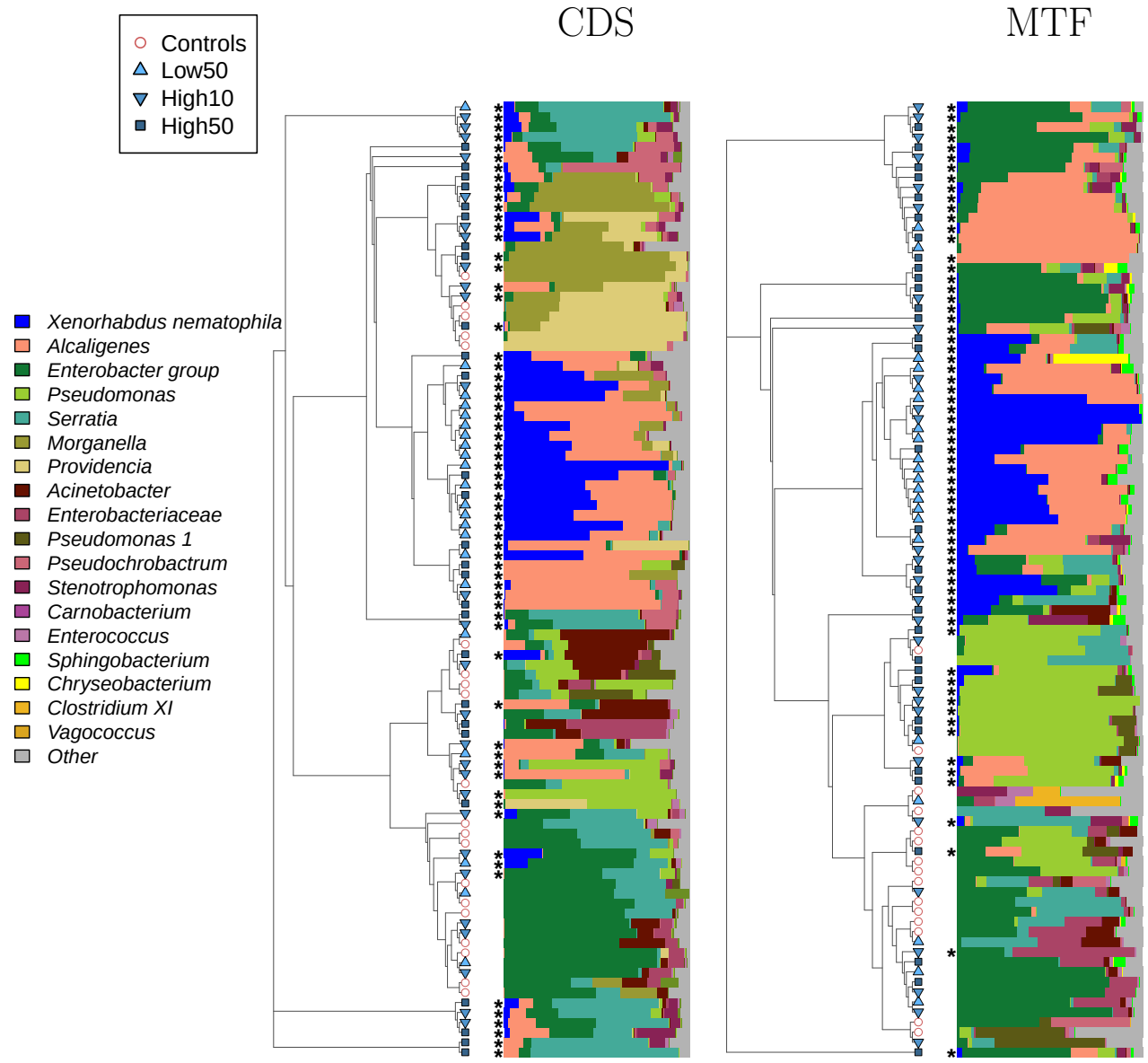


Figure 2: Community composition for each insect cadaver. Each dot/leaf represents one insect, and the neighbor-joining tree represents the Bray-Curtis distance between insects (with insects from each site being represented in separate trees). Coloured bars represent the relative abundance of OTUs in each insect cadaver. Each color correspond to one genus. Low abundance OTUs have been grouped in the "Other" category. Stars represent samples where *Xenorhabdus* have been detected either by metabarcoding or *tonB* receptor PCR

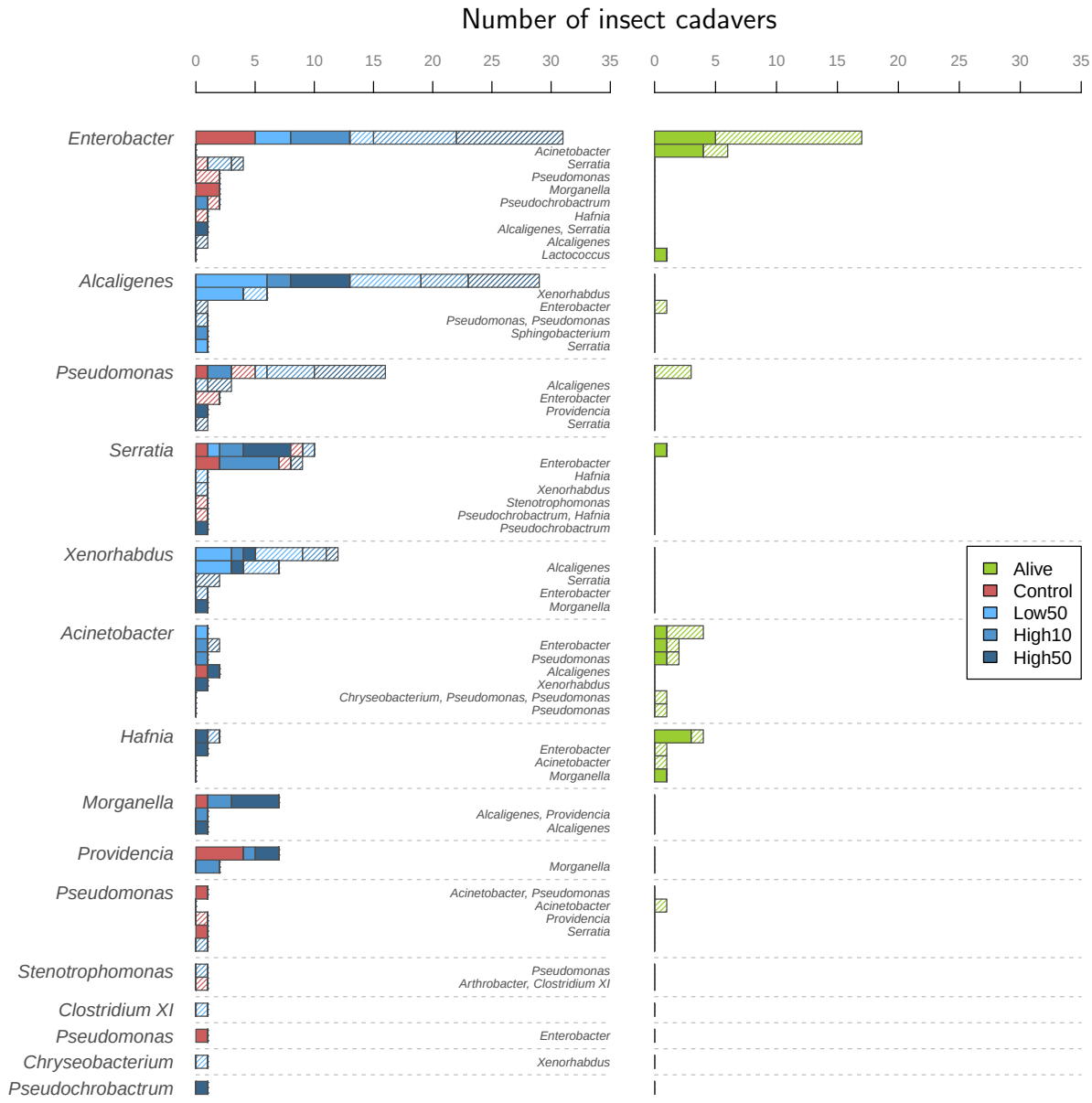


Figure 3: **Distribution of the dominant OTUs among treatments.** For each sample, we sorted OTUs by relative abundance and defined the dominant OTUs as OTUs needed to reach 50 % of the sample reads. Each block represents one dominant OTU, and bars represent insects where this OTU is the most abundant. This most abundant OTU can reach 50 % of samples reads alone, which corresponds to the first line of each block. The other lines of the blocks are the cases were the most abundant OTU dominates together with other OTUs which are indicated on each line.

and *Hafnia*, which can be found dominant in every treatments, as well as alive insects.

(ii) Some OTUs from the host microbiota did not dominate the community when insects are alive, but become dominant in cadavers. This is the case of *Serratia*, *Morganella* and *Providencia*. These OTUs have not been brought by nematodes as they can be dominant in the cadavers of uninfected insects. Interestingly, *Morganella* and *Providencia* are only present in one site (CDS), which shows that they are specific to a sampling site.

(iii) Some OTUs are only dominant only in dead insects that were infected. Two OTUs fall in this category, *Xenorhabdus* and *Alcaligenes*. They are brought by nematodes and manage to dominate the community after host death. Figure 3 clearly shows that communities dominated by *Alcaligenes* are more frequent than communities dominated by *Xenorhabdus*.

4 Conclusion

These preliminary results first confirm that *Xenorhabdus* does not necessarily dominates the bacterial community in the host cadaver at the time point of re-association with the vector. However, we found that the number of *Xenorhabdus* reads was higher for the treatment that initially brought the lower dose of bacteria. This can seem counter intuitive, but our guess is that infections with Low10 treatment were delayed compared to High10 and High50 treatments. Indeed, due to the lower number of bacteria that were brought by nematodes, insects probably died later. Thus, at the moment we analyzed bacterial community composition, *Xenorhabdus* from Low10 treatment could still be in an early phase of infection, while populations of other treatments had already declined, maybe because of nutrient depletion.

These first results also suggest that many different bacteria can dominate the community inside the host cadaver to the detriment of *Xenorhabdus*. These bacteria can either be dominant bacteria from the host microbiota, or less abundant bacteria from insect guts which were brought by soil, and were thus probably transient in the host. Finally, some dominant bacteria such as *Alcaligenes* can come from the vector microbiota. This suggests that, some competitors can take advantage of the killing provided by *Xenorhabdus-Steinernema* pair to multiply, and potentially out compete *Xenorhabdus*.

We do not know for now if the dominance of *Xenorhabdus* in the cadaver 10 days after infection is related or not to the efficiency of *Xenorhabdus* transmission by the next generation of nematodes. If transmission can indeed be predicted from the density of *X. nematophila*, our results suggest that *Xenorhabus* transmission rate should be quite low in nature. The unexpectedly high percentage of infections where we found no *X. nematophila* at all suggests that transmission of *Xenorhabdus* could fail in many infections, even if the insect has been killed and the nematodes have reproduced.

It may be that nematodes can always re-associate with *X. nematophila*, even when it

is found in really low frequency in the insect cadaver. The capacity of *Steinernema* to capture *X. nematophila* cells even when they are infrequent is suggested by the highly specific recognition mechanism involved in the re-association. But previous results suggest that the limiting factor for transmission is nematode reproduction. We therefore need to quantify nematode reproduction before we can understand how the composition of the bacterial community inside insect cadavers may impact the transmission of *X. nematophila*.

General conclusions and perspectives

1 Phenotypic heterogeneity in *X. nematophila*

1.1 A comparison of *Photorhabdus luminescens* and *X. nematophila* variants

The emergence of phenotypic variants during prolonged *in vitro* culture is known for decades in both *Xenorhabdus* and its sister genus *Photorhabdus* (Akhurst 1980). These *P. luminescens* variants, called secondary variants, share some features with group 2 variants of *Xenorhabdus* (Boemare et al. 1988; Boemare et al. 1997). The mechanisms responsible for their production is not yet fully understood, although the HexA regulator has been suggested to be involved (Joyce et al. 2003; Lanois et al. 2011; Langer et al. 2017). Interestingly, in *X. nematophila*, the transcription of *hexA* is under the control of Lrp (Engel et al. 2017) which suggests that the production of secondary variants in *X. nematophila* and in *P. luminescens* involves homologous regulatory networks.

Similarly to *X. nematophila*, again, *P. luminescens* produces a third type of variant. But while we have not identified the mechanism by which group 3 variants are produced in *X. nematophila*, their molecular origin is well understood in *P. luminescens*: these variants are produced by a mechanism which involves the production of the Mad pilus. Cells expressing these pili are able to persist in the maternal nematode intestine and consequently to colonize the IJ offspring that develop in the maternal body (Somvanshi et al. 2012). Within the cadaver of insect host, two forms of *P. luminescens* have been found. The P (Pathogenic) form of *P. luminescens* expresses many virulence factors which are necessary to kill the insect host, but does not express Mad pili which makes it unable to re-associate to the nematode vector. Conversely, M (Mutualistic) form does express Mad pili, and is thus the only form to be transmitted by nematodes, but is unable to kill the insect host. Genes controlling virulence and genes controlling the production of Mad pili are both under the control of the P_{mad} promoter. This promoter can be inverted by two invertases, which causes the ON/OFF switch of virulence and pilus phenotypes (Somvanshi et al. 2012). From that prospect, the M form of *P. luminescens* perfectly matches the classic definition of phase variants.

Contrarily to what is documented in *P. luminescens*, the three variants we have described in *X. nematophila* can re-associate with *S. carpocapsae* IJs. We also found no indications that one of these variants could be a phase variant (i.e. we found no specific reversible genetic or epigenetic mechanisms responsible for the production of these variants). In the specific case of group 2 variants, our results together with that of other teams (Cowles et al. 2007; Hussa et al. 2015) clearly indicate that mutations in the regulatory gene *lrp*, followed by strong selection during prolonged culture, are the cause of variation. Group 2 variants are in fact simply *lrp* defective mutants, which seem to be advantageous during late stationary phase because they have a reduced metabolism (they form small cells, and do not produce many of the secondary metabolites the group 1 variants excrete). These mutants can revert and recover some of the group 1 variant phenotypes, but we never observed that the non-mutated sequence of *lrp* was restored. This also suggests that the classical classification of primary and secondary variants (referred as group 1 and group 2 in this manuscript) is inaccurate. In fact, a continuum of phenotypes probably coexist during infection.

1.2 Selection of variants in *X. nematophila*: with- and between-hosts selective pressures

We found that variants from group 2 are usually advantaged in prolonged *in vitro* culture, and in aged insect cadavers. This seems to correspond to a **G**rowth **A**dvantage in **S**tationary **P**hase phenotype, well described in *E. coli*[1] (Zinser et al. 2000; Zinser et al. 2004; Finkel 2006). These GASP variants should thus increase in frequency during infection while nutrients are decreasing, due to within-host selective pressures. On the other hand, GASP variants impair nematode reproduction so that they are less transmitted than other variants. They should be counter-selected during transmission phase, due to between-hosts selective pressures. It is thus possible that GASP variants constantly appear due to *lrp* mutations during *X. nematophila* multiplication, that these GASP variants sometimes increase in frequency within some insects, but that they never reach high frequency in the global population[2] of *X. nematophila* because they fail to be transmitted.

One drawback of this reasoning is that nematode reproduction is impaired by GASP variants only when they are the only phenotypic form present during early infection. Indeed, when GASP variants are co-injected with non-GASP variants, we found that they had no detectable impact on transmission. GASP variants could therefore increase in frequency in the infection, even out compete non-GASP variants, and still be transmitted as long as non-GASP variants were present during the early stages of infection. This would

1. Interestingly, some GASP phenotypes in *E. coli* have been found to arise after a mutation in *lrp* (Zinser et al. 2004)

2. Here I mean "population" from its ecological definition, i.e. the population of *X. nematophila* at the ecosystem level, opposed to the population of *X. nematophila* in one insect used so far in the manuscript.

suggest that the between-host selective pressure imposed by nematode reproduction on GASP variants is weak.

And yet, *X. nematophila* extracted from nematodes sampled in the wild are, to my knowledge, always reported to be non-GASP variants. Other works from our lab suggest that another type of within-host selective pressure, that have nothing to do with transmission by nematodes, could be responsible from this situation. GASP variants do not indeed multiply well inside the insect in the presence of a competitor. If they are co-injected with, for example, *Aspergillus flavus* which is able to multiply in host cadavers, they perform worse than non-GASP variants. The presence of such competitors in nature could explain why GASP variants do not seem to be the majority form of *X. nematophila*. Although completely speculative, the same reasoning can be applied to bacteriocin-mediated competition between different strains of *X. nematophila*, or different species of *Xenorhabdus* (Hawlena et al. 2010b; Bashey et al. 2013). In any case, within-host selection is probably different in nature from what we observed in lab condition, and could explain why GASP variants are never found in nematodes.

1.3 What's next?

Before trying to further understand the impact *lrp* mutants can have on *Steinernema-Xenorhabdus* life cycle, we must be sure that they can arise in natural conditions. We have observed *lrp* mutants *X. nematophila* to arise from group 1 variants both *in vitro* and after injection in an insect. But we should also try to reproduce these experiments by initiating infections with nematodes that carry group 1 variants. Extracting bacteria from insect cadavers at different time points would allow us to describe the complete kinetic of variants apparition, from the time *X. nematophila* is brought by nematodes and until it re-associates to its vector. I expect to obtain the same results as described previously, but with the certitude that we are observing the right selective forces, taking into account the molecular dialog between bacteria and nematodes, other bacteria brought by nematodes etc... However, if we do not observe phenotypic diversification in these conditions, we could probably conclude that interactions between *Xenorhabdus* and the nematodes and/or its microbiota counter selects variants.

Another potential limitation of the experiments we present in this thesis, is that we used lab reared *Galleria mellonella* as an insect host. This insect is particularly susceptible to infection, and has probably an impoverished microbiota. I therefore suggest that experiments that aim at describing the kinetic of variants apparition should also be replicated using insects acclimatized to soil, as we described in Chapter 2. Experiments performed in these conditions should allow to study how the interactions between *Xenorhabdus* and other bacteria brought by nematodes and present in the host microbiota, both impact the phenotypic diversification of *X. nematophila* during the course of

infection. If ever we observe phenotypic diversification in insects that are not acclimatized to soil, but that this diversification does not occur in insects soil-acclimatized, we could probably conclude that the interactions between *X. nematophila* and microbes from the soil and the host microbiota counter select variants. This would explain why group 2 variants are not found in nematodes collected on the field.

If conversely we do observe diversification in the experimental conditions proposed above, we should then further investigate the selective pressures that are responsible for their appearance. We have documented a rapid and repeated production of variants of different kinds during prolonged culture or infections. Compared to group 1 variants, group 2 and group 3 variants have in common that they better resist to late stationary phase growth conditions, suggesting that their metabolism is modified. We also showed that these variants appear and stay in mixture in *in vitro* culture, suggesting a form of frequency dependent selection which could stabilize mixtures of variants with contrasted metabolisms.

A further step to better understand this metabolic diversification in populations of *X. nematophila* would be to refine the genotypic and phenotypic description of the collection of variants we have used in this study. To do so, we could perform RNA sequencing on pure cultures of variants, for example during exponential and stationary phase. This have been done on a *lrp* mutant that probably falls into our description of group 2 variants (Cowles et al. 2007), but we could expect that mutations appearing in the two active domains of Lrp have different impacts. Moreover, this RNA sequencing needs to be done on the new type of variants (group 3) we described. Overall, it would allow to have a better understanding of phenotypic diversification, in term of genes expression and regulation. Then, the whole genome of each variant should be sequenced to locate potential mutations other than in *lrp* gene. These two approaches combined should allow us to understand which mechanisms are responsible for the production of group 3 variants, and for partial phenotypic reversion, which are observed, but never associated with a reversion of the mutations we observed in *lrp*.

If metabolic diversification really occurs during infection in *X. nematophila*, and if this diversification does not involve the sole *lrp* regulator, we should probably not base our future work on the collection of variants we have used so far. These variants were indeed first screened using a single phenotypic trait: the color of colonies on a medium containing bromothymol blue. It is therefore highly probable that we have missed a large part of the phenotypic diversity that was present in late culture or late infections of *X. nematophila*. To investigate the genes under diversifying selection during late culture, we could instead replicate our experiments of *in vitro* evolution of *X. nematophila* and perform metagenomic sequencing at various time points of the experiment. Proceeding this way, we should be able to identify genes or genomic regions that become polymorphic due to diversifying selection. In principle, the same sort of experiments could be performed

in vivo; but DNA extract of *X. nematophila* would then be mixed with that of the insect and of other microbes present inside the infection. It may therefore be more efficient to run these experiments *in vivo* so that candidate gene can first be identified, and then to run *in vivo* experiments that would aim at confirming that these genes are targets of diversifying selection.

2 The potentially underestimated role of host and vector microbiota in *Xenorhabdus* life cycle

2.1 *Xenorhabdus nematophila* does not dominate the bacterial community inside insect cadavers

Most studies on entomopathogenic nematodes have focused on *Xenorhabdus* species. In fact *Xenorhabdus* has been considered so far as the sole symbiont of *Steinernema*, and the potential role of other bacteria that nematodes could carry has been largely neglected. Similarly, the impact the gut microbiota of insects could have on the life cycle of *Steinernema-Xenorhabdus* pairs has also rarely been investigated. One reason for this is that insects used in lab experiment are rarely soil-dwelling insects, and are reared on artificial medium, so that their gut microbiota is usually simplified. Moreover, before the development of next-generation sequencing methods, the study of bacterial communities depended on our ability to cultivate bacterial species.

Another reason that could explain the fact that bacteria from the host microbiota have been overlooked so far is that *Xenorhabdus* produces a lot of antimicrobial molecules. This led most people to take as granted that *X. nematophila* dominates the bacterial community inside insect cadavers, other microbes having only a minor impact on *Steinernema-Xenorhabdus* life cycle. We found that, contrary to our expectations, *Xenorhabdus* seldom dominates bacterial communities inside insects at the time-point at which it should re-associate with the nematode vector. In many infections, microbes other than *Xenorhabdus*, which may come either from the nematode or from the insect microbiota, seem to have taken advantage of the host killing to grow and exploit the host cadaver.

2.2 *Alcaligenes faecalis*, an artificial symbiont?

A striking pattern is that we found *Alcaligenes faecalis* in many of the insects cadavers we analyzed, often in higher relative abundance than *Xenorhabdus*. We found clear evidences that *A. faecalis* is brought by nematodes together with *Xenorhabdus*, which raises some questions about its role in the symbiosis. It may be, for example, that *A. faecalis* is responsible of part of the virulence that has so far been attributed to *Xenorhabdus*. *A. faecalis* could then be considered as yet another symbiont of *Steinernema*. This hypothesis is strengthen by our observation that *A. faecalis* is associated with all three nematode strains, as expected if indeed *A. faecalis* had co-evolved with *Steinernema*. Still, we observed a single *A. faecalis* OTU in our experiments, while one *A. faecalis* strain for each species of *Steinernema* would be expected if really co-evolution had occurred between the two taxa. Of course, this observation does not rule out the possibility that *A. faecalis* has co-evolved with *Steinernema* as it may well be that our single OTU gathers several

strains. Another scenario, though, which is to my opinion more likely, would be that this bacteria was present in the lab insects that have been used to maintain nematodes strains. Lab conditions would then have artificially enforced the association of *A. faecalis* with several species of *Steinernema*. If this hypothesis is correct, it nicely demonstrates that *Steinernema* can easily embark bacteria other than *Xenorhabdus* and suggest that they have a beneficial (or at least neutral) effect on nematodes reproduction in lab conditions, although Poinar (1988) found that *A. faecalis* has a negative effect on nematodes from the *Heterorhabditis* genus.[3] It would thus bring some interesting questions about the formation of long lasting interaction (either commensal or mutualistic) between nematodes and bacterial species when we modify their natural life cycle. These questions could also be of great interest in biocontrol. Indeed, for this application, IJ production is done in fermenters, where disequilibrium in bacterial communities may highly impact the nematode reproduction.

2.3 What's next?

The first thing that needs to be done, to my opinion, is to evaluate how of the dominance of *Xenorhabdus* in the bacterial community inside insects cadavers relates to its fitness, i.e. to its ability to be transmitted by nematodes. This can be a tricky thing to investigate, as describing the bacterial community in insect cadavers is destructive. Therefore it is not possible to measure transmission and community composition on the same insect. Moreover, the important inter-individual variability of the community composition in cadavers makes it difficult to relate *Xenorhabus* dominance and *Xenorhabdus* transmission measured on distinct individuals, even in very controlled conditions.

As a first step, we are currently performing digital droplet PCR (ddPCR) on two markers, that are specific to *Xenorhabdus* and *Steinernema* respectively. This should allow us to quantify first the number of *Xenorhabdus* cells and second some proxy of the nematode biomass inside insects, at the moment we performed the bacterial community description. The quantification of a nematode-specific marker is far from having an estimate of transmission: the only nematode stage that will transmit *Xenorhabdus* is IJs, and this technique cannot distinguish developmental stages that can be found together in the cadaver. Moreover, the size, and thus the associated biomass, of nematodes vary a lot between developmental stages. Nevertheless, this quantification of nematodes biomass will provide us some clues about the amount of nematodes present in the insect cadaver. This will be a rough estimation of nematode reproduction within insect cadaver, which we know to relate to *Xenorhabdus* transmission. We will thus be able to confirm or refute

3. It is interesting to note that in this paper, *A. faecalis* is considered as a contaminant, because at that time, entomopathogenic nematodes were considered as monoxenic, i.e. only carrying one species of symbiotic bacteria. We now know from DGIMI lab work that nematodes in fact has their own microbiota, and thus carry numerous bacterial species in addition to their symbiont (Ogier and Pagès et al. in prep).

the hypothesis that the more *Xenorhabdus* we found in the community, the best nematodes reproduce. If nematodes reproduce well in insects and manage to re-associate with their symbiont even when *Xenorhabdus* is in low frequency, it could led us to question the actual role of the antimicrobial molecules produced by *Xenorhabdus* described so far. For example, they could be implicated into the molecular dialog between *Xenorhabdus* and *Steinernema*, instead of interactions with *Xenorhabdus* microbial competitors.

Another step which seems logical to me would be to measure the impact of some of the bacteria we have identified in our experimental infections, as well as bacteria described in nematodes microbiota (project currently carried out in DGIMI lab, Ogier and Pagès, in prep.) on *Steinernema-Xenorhabdus* life cycle. The case of *A. faecalis* deserves particular attention, as we found it to be brought in insects by all the nematodes strains used in this study. Testing its impact on *Steinernema-Xenorhabdus* life cycle would first require to clarify its natural occurrence in nematodes. One difficulty here is that nematodes are usually sampled from nature using lab-reared insect as traps (Emelianoff et al. 2008b). We so far never had a chance to observe nematodes directly sampled from soil, but instead studied their offspring after a passage in a non-natural host. Assessing the association of *Steinernema* and *A. faecalis* in natural environment would therefore require the use of insect traps that do not carry *A. faecalis* in their gut. If this is feasible, we could then perform a complete microbiota analysis of nematodes sampled this way. Preliminary results on a freshly sampled strain of *S. carpocapsae* suggest that *A. faecalis* is absent from the nematodes microbiota (Ogier and Pagès in prep.). If confirmed on more field samplings, this result would indicate that the association between *A. faecalis* and *Steinernema* is indeed a lab artifact.

We could then further investigate the impact of other members of the nematode microbiota such as (e.g. *Pseudomonas, Stenotrophomonas, Alcaligenes, Ochrobactrum, Pseudochrobactrum, Brevundimonas, Achromobacter* (Ogier and Pagès in prep.)) on *Xenorhabdus-Steinernema* life cycle. To do so, we should make some *S. carpocapsae* strains that we artificially re-associate with *X. nematophila* or a mixture of *Xenorhabdus* and other members of the nematode microbiota. Sterile eggs of *S. carpocapsae* can hatch, develop and reproduce on lawns of *X. nematophila* bacterium in a petri dish (Vivas et al. 2001). IJs produced this way carry the *X. nematophila* strain from the lawn. We could thus also produce *S. carpocapsae* strains raised on lawns of a mixture of *X. nematophila* and/or other bacteria. The resulting IJs should then be used to infect insects. Doing so, we could measure and compare nematode reproduction and bacterial transmission R_0 with and without other members of the host microbiota associated to nematodes. We could also describe the bacterial community found in insect cadavers according to the set of bacteria associated with the nematodes. This could allow to identify some competitive interactions, or synergy between members of the nematode microbiota and the *Xenorhabdus* symbiont.

Open questions on heterogeneity in infections and mutualism stability

We showed in this work that *Steinernema-Xenorhabdus* infections are heterogeneous in several ways. First, the symbiont *Xenorhabdus* can evolve during infection, and produce sub-populations with contrasted phenotypic characteristics. Second, it seems to compete with a complex microbial community, including member of the host and the vector microbiota. These results both raise some questions about the stability of the mutualistic interaction between *Steinernema* and *Xenorhabdus*.

Mutants that arise during infection do not pay the cost of producing antimicrobial compounds and other exo-enzymes, but can benefit from those produced by non-mutants, and be transmitted by vectors. Although the definition of cheaters is complex and debated (Jones et al. 2015), the mutants we identified can at least in part fall in the broad definition of cheaters. The advantage of cheating poses a fundamental and long recognized problem to the evolutionary persistence of mutualisms (Axelrod et al. 1981). In the case of *X. nematophila*, how does the *Steinernema-Xenorhabdus* mutualism remain stable in spite of cheater-like mutants arising repeatedly during infections? Would the fact that mutants are not transmitted when they initiate the infection be sufficient to prevent them from invading the *Xenorhabdus* population at the community level? An interesting link could perhaps be made about how balance between within-host and between-host selective pressures probably stabilizes *Steinernema-Xenorhabdus* mutualism.

The fact that some bacteria from the nematode microbiota seem to outcompete *Xenorhabdus* during infection also questions the stability of *Steinernema-Xenorhabdus* interactions. In this circumstances, *Xenorhabdus* could end up at such low densities in the insect cadaver that its vector cannot re-associate properly with it. In the meanwhile, some other bacteria such as *Alcaligenes* highly dominate the community, and seem to be well transmitted by the nematode vector. What would happen if *Xenorhabus* promoted nematodes reproduction within insect cadavers but was not competitive enough and therefore failed to be transmitted to the benefit of other bacteria? The molecular recognition and highly specific re-association between *Steinernema* and *Xenorhabdus* is a sign that the mutualism between *Steinernema* and *Xenorhabdus* has been stable for a long time. This could suggest that some selective pressures yet to be identified stabilize this symbiosis.

These questions of mutualism stability in the context of infection heterogeneity are beyond the scope of this thesis, but could be interesting to investigate in future works.

Bibliography

Ackermann, M. (2015). "A functional perspective on phenotypic heterogeneity in microorganisms". *Nat. Rev. Microbiol.* 13.8, pp. 497–508.

Akhurst, R. (1980). "Morphological and functional dimorphism in *Xenorhabdus* spp bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*". *J. Gen. Microbiol.* 121, pp. 303–309.

Alizon, S., J. C. de Roode, and Y. Michalakis (2013). "Multiple infections and the evolution of virulence". *Ecol. Lett.* 16.4, pp. 556–567.

Axelrod, R. and W. Hamilton (1981). "The evolution of cooperation". *Science* 211.4489, pp. 1390–1396.

Bashey, F., S. K. Youg, H. Hawlena, and C. M. Lively (2012). "Spiteful interactions between sympatric natural isolates of *Xenorhabdus bovienii* benefit kin and reduce virulence". *J. Evolution. Biol.* 25.3, pp. 431–437.

Bashey, F., H. Hawlena, and C. M. Lively (2013). "Alternative paths to success in a parasite community: within-host competition can favor higher virulence or direct interference". *Evolution: International Journal of Organic Evolution* 67.3, pp. 900–907.

Belizário, J. E. and M. Napolitano (2015). "Human microbiomes and their roles in dysbiosis, common diseases, and novel therapeutic approaches". *Front. Microbiol.* 6.

Bell, A. S., J. C. Roode, D. Sim, and A. F. Read (2006). "Within-host competition in genetically diverse malaria infections: parasite virulence and competitive sucess". *Evolution* 60.7, pp. 1358–1371.

Bertoloni Meli, S. and F. Bashey (2018). "Trade-off between reproductive and anti-competitor abilities in an insect–parasitic nematode–bacteria symbiosis". *Ecology and evolution* 8.22, pp. 10847–10856.

Boemare, N., J. Thaler, and A. Lanois (1997). "Simple bacteriological tests for phenotypic characterization of *Xenorhabdus* and *Photorhabdus* phase variants". *Symbiosis* 22, pp. 167–175.

Boemare, N. (2002). "Systematics of *Photorhabdus* and *Xenorhabdus*". *Entomopathogenic nematology*, p. 35.

Boemare, N. and R. Akhurst (1988). "Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp.(Enterobacteriaceae)". *J. Gen. Microbiol.* 134.3, pp. 751–761.

Boissière, A., M. T. Tchioffo, D. Bachar, L. Abate, A. Marie, S. E. Nsango, et al. (2012). "Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and nteractions with *Plasmodium falciparum* infection". *PLoS Pathog.* 8.5, pp. 1–12.

Bordenstein, S. R. and K. R. Theis (2015). "Host biology in light of the microbiome: ten principles of holobionts and hologenomes". *PLoS Biol.* 13.8, e1002226.

Broderick, N. A., N. Buchon, and B. Lemaitre (2014). "Microbiota-induced changes in *Drosophila melanogaster* host gene expression and gut morphology". *MBio* 5.3, e01117–14.

Brugiroux, S., M. Beutler, C. Pfann, D. Garzetti, H.-J. Ruscheweyh, D. Ring, M. Diehl, S. Herp, Y. Lötscher, S. Hussain, et al. (2016). "Genome-guided design of a defined mouse microbiota that confers colonization resistance against *Salmonella enterica* serovar Typhimurium". *Nat. Microbiol.* 2, p. 16215.

Caccia, S., I. Di Lelio, A. La Storia, A. Marinelli, P. Varricchio, E. Franzetti, N. Banyuls, G. Tettamanti, M. Casartelli, B. Giordana, et al. (2016). "Midgut microbiota and host immunocompetence underlie *Bacillus thuringiensis* killing mechanism". *P. Natl. Acad. Sci. USA* 113.34, pp. 9486–9491.

Caldas, C., A. Cherqui, A. Pereira, and N. Simoes (2002). "Purification and characterization of an extracellular protease from *Xenorhabdus nematophila* involved in insect immunosuppression". *Appl. Environ. Microbiol.* 68.3, pp. 1297–1304.

Chapuis, E., V. Emelianoff, V. Paulmier, N. Le Brun, M. Sicard, J.-B. Ferdy, et al. (2009). "Manifold aspects of specificity in a nematode–bacterium mutualism". *J. Evolution. Biol.* 22.10, pp. 2104–2117.

Chapuis, E., A. Arnal, and J.-B. Ferdy (2012). "Trade-offs shape the evolution of the vector-borne insect pathogen *Xenorhabdus nematophila*". *P. Roy. Soc. B-Biol. Sci.* 279.1738, pp. 2672–2680.

Chaston, J. M., K. E. Murfin, E. A. Heath-Heckman, and H. Goodrich-Blair (2013). "Previously unrecognized stages of species-specific colonization in the mutualism between *Xenorhabdus* bacteria and *Steinernema* nematodes". *Cell. Microbiol.* 15.9, pp. 1545–1559.

Chen, G., Y. Zhang, J. Li, G. Dunphy, Z. Punja, and J. Webster (1996). "Chitinase activity of *Xenorhabdus* and *Photorhabdus* species, bacterial associates of entomopathogenic nematodes". *J. Invertebr. Pathol.* 68.2, pp. 101–108.

Cirimotich, C. M., J. L. Ramirez, and G. Dimopoulos (2011). "Native microbiota shape insect vector competence for human pathogens". *Cell Host Microbe* 10.4, pp. 307–310.

Cowles, K. N., C. E. Cowles, G. R. Richards, E. C. Martens, and H. Goodrich-Blair (2007). "The global regulator Lrp contributes to mutualism, pathogenesis and phenotypic variation in the bacterium *Xenorhabdus nematophila*". *Cell. Microbiol.* 9.5, pp. 1311–1323.

Crawford, J. M., C. Portmann, X. Zhang, M. B. Roeffaers, and J. Clardy (2012). "Small molecule perimeter defense in entomopathogenic bacteria". *P. Natl. Acad. Sci. USA* 109.27, pp. 10821–10826.

Dennison, N. J., N. Jupatanakul, and G. Dimopoulos (2014). "The mosquito microbiota influences vector competence for human pathogens". *Curr. Opin. Insect Sci.* 3. Vectors and medical and veterinary entomology, pp. 6–13.

Didelot, X., A. S. Walker, T. E. Peto, D. W. Crook, and D. J. Wilson (2016). "Within-host evolution of bacterial pathogens". *Nat. Rev. Microbiol.* 14.3, p. 150.

Dowds, B. C. and A. Peters (2002). "Entomopathogenic Nematology". *Entomopathogenic nematology*. Ed. by R. Gaugler. CABI. Chap. Virulence mechanisms, pp. 79–98.

Embers, M. E., R. Ramamoorthy, and M. T. Philipp (2004). "Survival strategies of *Borrelia burgdorferi*, the etiologic agent of Lyme disease". *Microbes Infect.* 6.3, pp. 312–318.

Emelianoff, V., E. Chapuis, N. Le Brun, M. Chiral, C. Moulia, and J.-B. Ferdy (2008a). "A survival-reproduction trade-off in entomopathogenic nematodes mediated by their bacterial symbionts". *Evolution* 62.4, pp. 932–942.

Emelianoff, V., N. Le Brun, S. Pagès, S. P. Stock, P. Tailliez, C. Moulia, and M. Sicard (2008b). "Isolation and identification of entomopathogenic nematodes and their symbiotic bacteria from Hérault and Gard (Southern France)". *J. Invertebr. Pathol.* 98.2, pp. 211–217.

Emelianoff, V., M. Sicard, N. Le Brun, C. Moulia, and J.-B. Ferdy (2007). "Effect of bacterial symbionts *Xenorhabdus* on mortality of infective juveniles of two *Steinernema* species". *Parasitol. Res.* 100.3, pp. 657–659.

Engel, P., W. K. Kwong, Q. McFrederick, K. E. Anderson, S. M. Barribeau, J. A. Chandler, R. S. Cornman, J. Dainat, J. R. de Miranda, V. Doublet, et al. (2016). "The bee microbiome: impact on bee health and model for evolution and ecology of host-microbe interactions". *MBio* 7.2, e02164–15.

Engel, Y., C. Windhorst, X. Lu, H. Goodrich-Blair, and H. B. Bode (2017). "The Global Regulators Lrp, LeuO, and HexA Control Secondary Metabolism in Entomopathogenic Bacteria". *Front. Microbiol.* 8, p. 209.

Evans, A. S. (1976). "Causation and disease: the Henle-Koch postulates revisited". *The Yale journal of biology and medicine* 49.2, p. 175.

Finkel, S. E. (2006). "Long-term survival during stationary phase: evolution and the GASP phenotype". *Nat. Rev. Microbiol.* 4.2, p. 113.

Forst, S. and B. Boylan (2002). "Characterization of the pleiotropic phenotype of an ompR strain of *Xenorhabdus nematophila*". *Antonie van Leeuwenhoek* 81.1, p. 43.

Garcia-Pastor, L., E. Puerta-Fernàndez, and J. Casadesùs (2018). "Bistability and phase variation in *Salmonella enterica*". *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*.

Givaudan, A., S. Baghdiguian, A. Lanois, and N. Boemare (1995). "Swarming and swimming changes concomitant with phase variation in *Xenorhabdus nematophilus*." *Appl. Environ. Microbiol.* 61.4, pp. 1408–1413.

Givaudan, A., A. Lanois, and N. Boemare (1996). "Cloning and nucleotide sequence of a flagellin encoding genetic locus from *Xenorhabdus nematophilus*: phase variation leads to differential transcription of two flagellar genes (*fliCD*)". *Gene* 183.1, pp. 243–253.

Goodrich-Blair, H. (2007). "They've got a ticket to ride: *Xenorhabdus nematophila-Steinernema carpocapsae* symbiosis". *Curr. Opin. Microbiol.* 10.3, pp. 225–230.

Hawlena, H., F. Bashey, and C. M. Lively (2010a). "The evolution of spite: population structure and bacteriocin-mediated antagonism in two natural populations of *Xenorhabdus* bacteria". *Evolution* 64.11, pp. 3198–3204.

Hawlena, H., F. Bashey, H. Mendes-Soares, and C. M. Lively (2010b). "Spiteful Interactions in a Natural Population of the Bacterium Xenorhabdus bovienii." *The American Naturalist* 175.3. PMID: 20095826, pp. 374–381.

Heungens, K., C. E. Cowles, and H. Goodrich-Blair (2002). "Identification of *Xenorhabdus nematophila* genes required for mutualistic colonization of *Steinernema carpocapsae* nematodes". *Mol. Microbiol.* 45.5, pp. 1337–1353.

Hussa, E. A., Á. M. Casanova-Torres, and H. Goodrich-Blair (2015). "The global transcription factor Lrp controls virulence modulation in *Xenorhabdus nematophila*". *J Bacteriol.* JB–00272.

Jones, E. I., M. E. Afkhami, E. Akçay, J. L. Bronstein, R. Bshary, M. E. Frederickson, K. D. Heath, J. D. Hoeksema, J. H. Ness, M. S. Pankey, et al. (2015). "Cheaters must prosper: reconciling theoretical and empirical perspectives on cheating in mutualism". *Ecol. Lett.* 18.11, pp. 1270–1284.

Joyce, S. A. and D. J. Clarke (2003). "A *hexA* homologue from *Photorhabdus* regulates pathogenicity, symbiosis and phenotypic variation". *Mol. Microbiol.* 47.5, pp. 1445–1457.

Jubelin, G., A. Lanois, D. Severac, S. Rialle, C. Longin, S. Gaudriault, and A. Givaudan (2013). "FliZ Is a global regulatory protein affecting the expression of flagellar and virulence genes in individual *Xenorhabdus nematophila* bacterial cells". *PLOS Genet.* 9.10, e1003915.

Kaiser, P., M. Diard, B. Stecher, and W.-D. Hardt (2012). "The streptomycin mouse model for *Salmonella* diarrhea: functional analysis of the microbiota, the pathogen's virulence factors, and the host's mucosal immune response". *Immunol. Rev.* 245.1, pp. 56–83.

Kim, I.-H., S. K. Aryal, D. T. Aghai, Á. M. Casanova-Torres, K. Hillman, M. P. Kozuch, E. J. Mans, T. J. Mauer, J.-C. Ogier, J. C. Ensign, et al. (2017). "The insect pathogenic bacterium *Xenorhabdus innexi* has attenuated virulence in multiple insect model hosts yet encodes a potent mosquitocidal toxin". *BMC genomics* 18.1, p. 927.

Koch, R. (1876). “The etiology of anthrax, based on the life history of *Bacillus anthracis*”. *Milestones in Microbiology, 1546 to 1940*. Ed. by T. D. Brock. ASM press 1999. Chap. The Germ Theory of Disease, pp. 89–95.

— (1882). “The etiology of tuberculosis”. *Milestones in Microbiology, 1546 to 1940*. Ed. by T. D. Brock. ASM press 1999. Chap. The Germ Theory of Disease, pp. 109–115.

Labandeira-Rey, M., J. Seshu, and J. T. Skare (2003). “The absence of linear plasmid 25 or 28-1 of *Borrelia burgdorferi* dramatically alters the kinetics of experimental infection via distinct mechanisms”. *Infect. Immun.* 71.8, pp. 4608–4613.

Langer, A., A. Moldovan, C. Harmath, S. A. Joyce, D. J. Clarke, and R. Heermann (2017). “HexA is a versatile regulator involved in the control of phenotypic heterogeneity of *Photorhabdus luminescens*”. *PLoS One* 12.4, e0176535.

Lanois, A., S. Pages, S. Bourot, A.-S. Canoy, A. Givaudan, and S. Gaudriault (2011). “Transcriptional analysis of a *Photorhabdus* sp. variant reveals transcriptional control of phenotypic variation and multifactorial pathogenicity in insects”. *Appl. Environ. Microbiol.* 77.3, pp. 1009–1020.

Lanois, A., J.-C. Ogier, J. Gouzy, C. Laroui, Z. Rouy, A. Givaudan, and S. Gaudriault (2013). “Draft genome sequence and annotation of the entomopathogenic bacterium *Xenorhabdus nematophila* strain F1”. *Microbiol.Res. Ann.* 1.3.

Lee, M.-M. and S. P. Stock (2010). “A multilocus approach to assessing co-evolutionary relationships between *Steinernema* spp.(Nematoda: Steinernematidae) and their bacterial symbionts *Xenorhabdus* spp.(γ-Proteobacteria: Enterobacteriaceae)”. *Systematic Parasitology* 77.1, pp. 1–12.

Lerner, B. W. and K. L. Lerner (2006a). “Koch’s Postulate”. *World of Microbiology and Immunology*. Ed. by B. Narins. Gale, pp. 326–228.

— (2006b). “Robert Koch”. *World of Microbiology and Immunology*. Ed. by B. Narins. Gale, pp. 326–228.

Lieberman, T. D., K. B. Flett, I. Yelin, T. R. Martin, A. J. McAdam, G. P. Priebe, and R. Kishony (2014). “Genetic variation of a bacterial pathogen within individuals with cystic fibrosis provides a record of selective pressures”. *Nat. Genet.* 46.1, p. 82.

Lieberman, T. D., J.-B. Michel, M. Aingaran, G. Potter-Bynoe, D. Roux, M. R. Davis Jr, D. Skurnik, N. Leiby, J. J. LiPuma, J. B. Goldberg, et al. (2011). “Parallel bacterial evolution within multiple patients identifies candidate pathogenicity genes”. *Nat. Genet.* 43.12, p. 1275.

Martens, E. C., K. Heungens, and H. Goodrich-Blair (2003). “Early colonization events in the mutualistic association between *Steinernema carpocapsae* nematodes and *Xenorhabdus nematophila* bacteria”. *J Bacteriol.* 185.10, pp. 3147–3154.

McMullen, J. G., R. McQuade, J.-C. Ogier, S. Pagès, S. Gaudriault, and S. Patricia Stock (2017). “Variable virulence phenotype of *Xenorhabdus bovienii* (γ-Proteobacteria: En-

terobacteriaceae) in the absence of their vector hosts". *Microbiology* 163.4, pp. 510–522.

Mideo, N. (2009). "Parasite adaptations to within-host competition". *Trends Parasitol.* 25.6, pp. 261–268.

Morales-Soto, N. and S. A. Forst (2011). "The xnp1 P2-like tail synthesis gene cluster encodes xenorhabdicin and is required for interspecies competition". *J Bacteriol.* 193.14, pp. 3624–3632.

Morgan, J., V. Kuntzelmann, S. Tavernor, M. Ousley, and C. Winstanley (1997). "Survival of *Xenorhabdus nematophilus* and *Photorhabdus luminescens* in water and soil". *J. Appl. Microbiol.* 83.6, pp. 665–670.

Narasimhan, S., N. Rajeevan, L. Liu, Y. O. Zhao, J. Heisig, J. Pan, R. Eppler-Epstein, K. DePonte, D. Fish, and E. Fikrig (2014). "Gut microbiota of the tick vector *Ixodes scapularis* modulate colonization of the Lyme disease Spirochete". *Cell Host Microbe* 15.1, pp. 58–71.

Ogier, J.-C., S. Pagès, G. Bisch, H. Chiapello, C. Médigue, Z. Rouy, C. Teyssier, S. Vincent, P. Tailliez, A. Givaudan, et al. (2014). "Attenuated virulence and genomic reductive evolution in the entomopathogenic bacterial symbiont species, *Xenorhabdus poinarii*". *Genome Biol. Evol.* 6.6, pp. 1495–1513.

Park, D., K. Ciezki, R. Van Der Hoeven, S. Singh, D. Reimer, H. B. Bode, and S. Forst (2009). "Genetic analysis of xenocoumacin antibiotic production in the mutualistic bacterium *Xenorhabdus nematophila*". *Mol. Microbiol.* 73.5, pp. 938–949.

Park, Y., E. E. Herbert, C. E. Cowles, K. N. Cowles, M. L. Menard, S. S. Orchard, and H. Goodrich-Blair (2007). "Clonal variation in *Xenorhabdus nematophila* virulence and suppression of *Manduca sexta* immunity". *Cell Microbiol.* 9.3, pp. 645–656.

Pasteur, L. (1857). "Report on the lactic acid fermentation (author's abstract)". *Milestones in Microbiology, 1546 to 1940*. Ed. by T. D. Brock. ASM press 1999. Chap. Spontaneous Generation and Fermentation, pp. 27–30.

— (1861). "On the organized bodies which exist in the atmosphere; examination of the doctrine of spontaneous generation". *Milestones in Microbiology, 1546 to 1940*. Ed. by T. D. Brock. ASM press 1999. Chap. Spontaneous Generation and Fermentation, pp. 43–48.

Peters, A. (1996). "The natural host range of *Steinernema* and *Heterorhabditis* spp. and their impact on insect populations". *Biocontrol Sci. Technol.* 6.3, pp. 389–402.

Poinar, G. O. (1988). "Morphological and behavioral abnormalities in *Heterorhabditis heliothidis* (Rhabditida) associated with the bacterium, *Alcaligenes faecalis*". *REV. NEMATOL.* 11.4, pp. 381–383.

Raymond, B., P. R. Johnston, C. Nielsen-LeRoux, D. Lereclus, and N. Crickmore (2010). "*Bacillus thuringiensis*: an impotent pathogen?" *Trends Microbiol.* 18.5, pp. 189–194.

Richards, G. R. and H. Goodrich-Blair (2009). "Masters of conquest and pillage: *Xenorhabdus nematophila* global regulators control transitions from virulence to nutrient acquisition". *Cell. Microbiol.* 11.7, pp. 1025–1033.

Richards, G. R., E. E. Herbert, Y. Park, and H. Goodrich-Blair (2008). "*Xenorhabdus nematophila* lrhA is necessary for motility, lipase activity, toxin expression, and virulence in *Manduca sexta* insects". *J Bacteriol.* 190.14, pp. 4870–4879.

Shao, Y., B. Chen, C. Sun, K. Ishida, C. Hertweck, and W. Boland (2017). "Symbiont-derived antimicrobials contribute to the control of the cepidopteran gut microbiota". *Cell Chem. Biol.* 24.1, pp. 66–75.

Shi, W., S. Xie, X. Chen, S. Sun, X. Zhou, L. Liu, P. Gao, N. C. Kyrpides, E.-G. No, and J. S. Yuan (2013). "Comparative genomic analysis of the endosymbionts of herbivorous insects reveals eco-environmental adaptations: biotechnology applications". *PLOS Genet.* 9.1, pp. 1–12.

Sicard, M., K. Brugirard-Ricaud, S. Pagès, A. Lanois, N. E. Boemare, M. Brehélin, and A. Givaudan (2004a). "Stages of infection during the tripartite interaction between *Xenorhabdus nematophila*, its nematode vector, and insect hosts". *Appl. Environ. Microbiol.* 70.11, pp. 6473–6480.

Sicard, M., J.-B. Ferdy, N. Le Brun, B. Godelle, N. Boemare, and C. Moulia (2004b). "When mutualists are pathogens: an experimental study of the symbioses between *Steinernema* (entomopathogenic nematodes) and *Xenorhabdus* (bacteria)". *J. Evolution. Biol.* 17.5, pp. 985–993.

Sicard, M., N. Le Brun, S. Pages, B. Godelle, N. Boemare, and C. Moulia (2003). "Effect of native *Xenorhabdus* on the fitness of their *Steinernema* hosts: contrasting types of interaction". *Parasitol. Res.* 91.6, pp. 520–524.

Sicard, M., J. Tabart, N. Boemare, J.-O. Thaler, and C. Moulia (2005). "Effect of phenotypic variation in *Xenorhabdus nematophila* on its mutualistic relationship with the entomopathogenic nematode *Steinernema carpocapsae*". *Parasitology* 131, pp. 687–694.

Singh, J. and N. Banerjee (2008). "Transcriptional analysis and functional characterization of a gene pair encoding iron-regulated xenocin and immunity proteins of *Xenorhabdus nematophila*". *J Bacteriol.* 190.11, pp. 3877–3885.

Singh, S., D. Orr, E. Divinagracia, J. McGraw, K. Dorff, and S. Forst (2015). "Role of secondary metabolites in establishment of the mutualistic partnership between *Xenorhabdus nematophila* and the entomopathogenic nematode *Steinernema carpocapsae*". *Appl. Environ. Microbiol.* 81.2, pp. 754–764.

Snyder, H., S. P. Stock, S.-K. Kim, Y. Flores-Lara, and S. Forst (2007). "New insights into the colonization and release processes of *Xenorhabdus nematophila* and the morphology and ultrastructure of the bacterial receptacle of its nematode host, *Steinernema carpocapsae*". *Appl. Environ. Microbiol.* 73.16, pp. 5338–5346.

Somvanshi, V. S., R. E. Sloup, J. M. Crawford, A. R. Martin, A. J. Heidt, K.-s. Kim, J. Clardy, and T. A. Ciche (2012). "A single promoter inversion switches *Photorhabdus* between pathogenic and mutualistic states". *Science* 337.6090, pp. 88–93.

Stecher, B., R. Denzler, L. Maier, F. Bernet, M. J. Sanders, D. J. Pickard, M. Barthel, A. M. Westendorf, K. A. Krogfelt, A. W. Walker, et al. (2012). "Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae". *Proceedings of the National Academy of Sciences* 109.4, pp. 1269–1274.

Stecher, B. and W.-D. Hardt (2008). "The role of microbiota in infectious disease". *Trends Microbiol.* 16.3, pp. 107–114.

Stewart, M. K., L. A. Cummings, M. L. Johnson, A. B. Berezow, and B. T. Cookson (2011). "Regulation of phenotypic heterogeneity permits *Salmonella* evasion of the host caspase-1 inflammatory response". *Proceedings of the National Academy of Sciences* 108.51, pp. 20742–20747.

Telfer, S., X. Lambin, R. Birtles, P. Beldomenico, S. Burthe, S. Paterson, and M. Begon (2010). "Species interactions in a parasite community drive infection risk in a wildlife population". *Science* 330.6001, pp. 243–246.

Thaler, J. O., S. Baghdiguian, and N. Boemare (1995). "Purification and characterization of xenorhabdicin, a phage tail-like bacteriocin, from the lysogenic strain F1 of *Xenorhabdus nematophilus*". *Appl. Environ. Microbiol.* 61.5, pp. 2049–2052.

Thaler, J.-O., B. Duvic, A. Givaudan, and N. Boemare (1998). "Isolation and entomotoxic properties of the *Xenorhabdus nematophilus* F1 lecithinase". *Appl. Environ. Microbiol.* 64, pp. 2367–2373.

van der Woude, M. W. (2006). "Re-examining the role and random nature of phase variation". *FEMS Microbiol. Lett.* 254, pp. 190–197.

— (2011). "Phase variation: how to create and coordinate population diversity". *Curr. Opin. Microbiol.* 14.2, pp. 205–211.

van der Woude, M. W. and A. J. Bäumler (2004). "Phase and antigenic variation in bacteria". *Clin. Microbiol. Rev.* 17.3, pp. 581–611.

Vigneux, F., B. F, M. Sicard, and C. M. Lively (2008). "Low migration decreases interference competition among parasites and increases virulence". *J. Evolution. Biol.* 21.5, pp. 1245–1251.

Vigneux, F., R. Zumbihl, G. Jubelin, C. Ribeiro, J. Poncet, S. Baghdiguian, A. Givaudan, and M. Brehélin (2007). "The xaxAB genes encoding a new apoptotic toxin from the insect pathogen *Xenorhabdus nematophila* are present in plant and human pathogens". *J. Biol. Chem.* 282.13, pp. 9571–9580.

Vivas, E. I. and H. Goodrich-Blair (2001). "*Xenorhabdus nematophilus* as a model for host-bacterium interactions: *rpoS* is necessary for mutualism with nematodes". *J Bacteriol.* 183.16, pp. 4687–4693.

Volgyi, A., A. Fodor, A. Szentirmai, and S. Forst (1998). "Phase variation in *Xenorhabdus nematophilus*". *Appl. Environ. Microbiol.* 64.4, pp. 1188–1193.

Weiss, B. and S. Aksoy (2011). "Microbiome influences on insect host vector competence". *Trends Parasitol.* 27.11, pp. 514–522.

Welte, C. U., R. M. de Graaf, T. J. van den Bosch, H. J. Op den Camp, N. M. van Dam, and M. S. Jetten (2016). "Plasmids from the gut microbiome of cabbage root fly larvae encode SaxA that catalyses the conversion of the plant toxin 2-phenylethyl isothiocyanate". *Environ. Microbiol.* 18.5, pp. 1379–1390.

Zhou, X., H. K. Kaya, K. Heungens, and H. Goodrich-Blair (2002). "Response of ants to a deterrent factor (s) produced by the symbiotic bacteria of entomopathogenic nematodes". *Appl. Environ. Microbiol.* 68.12, pp. 6202–6209.

Zinser, E. R. and R. Kolter (2000). "Prolonged stationary-phase incubation selects for *lrp* mutations in *Escherichia coli* K-12". *Journal of Bacteriology* 182.15, pp. 4361–4365.

Zinser, E. R. and R. Kolter (2004). "*Escherichia coli* evolution during stationary phase". *Res. Microbiol.* 155.5, pp. 328–336.

Appendices

Supplementary material for Chapter 1: "Prolonged culture and long lasting infections select for poorly transmitted bacterial variants"

All supplementary material and datasets mentioned in the text can be downloaded here: `https://dl.univ-tlse3.fr/filez/vw6pl`

Name	Group	Culture ID	Day of growth	Mutation position	Mutation type
G1#12	1	9	3		
G1#20	1	7	7		
G1#21	1	7	7		
G1#23	1	9	7		
G1#26	1	11	7		
G1#30	1	12	7		
G1#35	1	1	13		
G1#38	1	2	13		
G1#42	1	3	13		
G1#43	1	3	13		
G1#44	1	5	13		
G1#46	1	5	13		
G1#51	1	6	13		
G1#56	1	8	13		
G1#59	1	8	13		
G2#18	2	7	7	55	Q $\rightarrow$ $*$
G2#19	2	7	7	55	Q $\rightarrow$ $*$
G2#24	2	11	7	120	R $\rightarrow$ C
G2#25	2	11	7	120	R $\rightarrow$ C
G2#28	2	12	7	120	R $\rightarrow$ H
G2#29	2	12	7	120	R $\rightarrow$ H
G2#31	2	12	7		
G2#32	2	1	13	55	Q $\rightarrow$ $*$
G2#36	2	2	13	53	FS
G2#37	2	2	13	53	FS
G2#39	2	2	13	53	FS
G2#40	2	3	13	124	dupl.
G2#41	2	3	13	124	dupl.
G2#45	2	5	13	55	Q $\rightarrow$ $*$
G3#22	3	9	7		
G3#27	3	11	7		
G3#47	3	5	13		
G3#48	3	6	13		
G3#9	3	9	3		

Table S1 – Description of the collection of variants. Day corresponds to the number of days of growth after which each variant was isolated. Mutation types: for SNPs that cause a substitution, the amino-acid change is given. Stars indicate the truncation of the protein. FS stands for frame shift, and dupli. for duplications.

Prolonged culture and long lasting infections select for poorly transmitted bacterial variants — Supplementary materials S2

November 30, 2018

In vitro kinetics of the 34 isolates of the collection

We assessed *in vitro* growth kinetics of the 34 isolates by measuring GFP fluorescence using a Synergy BioTeK spectrophotometer. More precisely, GFP-mediated fluorescence was estimated by measuring emissions at 535nm after excitation at 485nm. In order to control for potential autofluorescence, we also measured emissions at 625nm and computed the log-transformed ratio of emissions at 535nm and 625nm. In the following, we will consider this quantity as an estimate of GFP emission intensity.

For each experiment, 24 hours precultures were first initiated from cryotubes and incubated in 200 μL LB in 96-wells clear-bottom microplates (Greiner) at 28°C with 20μg Kanamycin per mL. Each of the 34 variants was randomly assigned to two to three positions in the plate and a single well filled with sterile culture medium was used as a negative control.

After 24 hours, precultures were centrifugated and rinsed in sterile LB. We then

measured GFP emission intensity and performed a dilution in order to compensate for differences in fluorescence among the wells of the microplate. From then, we transferred 20μL of a 10^{-3} dilution of each preculture into approx. 180μL of fresh LB with Kanamycin added. The culture microplate we obtained was then placed in the spectrophotometer, at 28°C with shaking, and measurements were performed every 15 minutes for 91 hours. We ran five replicate experiments.

In addition to fluorescence measurements, we estimated bacterial density by spreading appropriate dilutions of cultures (typically 10^{-5} or 10^{-6}) onto NBTA plates with 50μg Kanamycin per ml. Colonies were counted after 48 hours incubation at 28°C. This was performed first at the onset of the experiment, in order to both check that cell densities were the same in all wells of the culture microplate and estimate inoculum size. This was also performed after the 91 hours of *in vitro* cultivation, so that we can check that differences in fluorescence do relate to differences in cell density. From the spectrophotometer measurements, we estimated the time at which we detected bacterial growth in each well. This was done by first estimating the average GFP intensity over the first 3 hours of the kinetics, a time at which bacteria have not yet multiplied, and adding an arbitrary value of five percent to this average value. We obtained this way a threshold intensity above which we consider that fluorescence is due to the presence of bacteria. For each well of the culture microplate, we then estimated the time at which the GFP intensity has reached the threshold value. We will further refer to this computation as time lag.

From the same experiments, we computed the average number of Colonies Forming Units (CFU) per mL, from counts on NBTA plates after the 91 hours of cultivation. In most cultures, number of CFU per mL could be estimated for two distinct dilutions. In this situation, we averaged the two estimates. We used these NBTA plates to estimate the proportion of red CFU for each of the 34 variants.

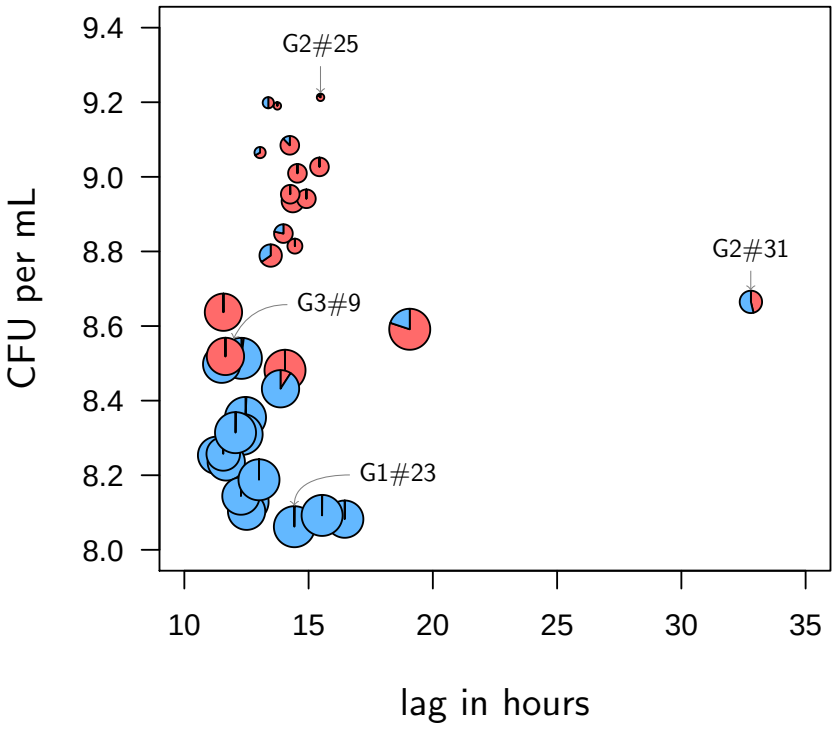


Figure S2-1: Lag in hours and density in log CFU per mL for each of the 34 isolates of the collection.

For each of the 34 isolates, following the procedure described above, we obtained from 12 to 15 estimates of time lag and from 9 to 12 estimates of CFU numbers. We summarized all these data by computing average values which are represented in the figure below. In this figure, G2#31 clearly stands apart as the isolate with the longest lag. Overall, this figure also illustrates that group 2 and to a lesser extent group 3 variants reach higher densities than group 1 variants. These estimations also demonstrate that group 2 isolates start growing later (Wilcoxon test: $p = 0.004$) but reach higher CFU per mL (Wilcoxon test: $p = 7.7e - 8$) than group 1 isolates. Group 3 variants also reach higher CFU per mL than group 1 (Wilcoxon test: $p = 0.00155$) but have a similar lag.

Prolonged culture and long lasting infections select for poorly transmitted bacterial variants— Supplementary materials S3

February 12, 2019

Integration of $P_{lac} - RBS - lrp$ in the chromosome of G2#25 and full phenotype restoration

1 Materials and methods

First, an *lrp* PCR fragment (including its own RBS but not promoter) is generated using primers L-PstI-RBS-lrp and R-BamHI-lrp and genomic DNA of *X. nematophila* F1 wild type as template. This amplicon was digested with PstI and BamHI and finally cloned under the control of P_{lac} promoter of the pBBR1-MCS1 vector (Kovach et al., 1994) by digestion with PstI and BamHI generating the pBB-P_{lac}-RBS-*lrp* plasmid. After digestion of pJQ200SK suicide vector with SalI and SpeI, three DNA fragments (i) PCR amplicon of glmS

from F1 (SpeI-AatII digested) (ii) PCR amplicon of the ATPase site from F1 (SacI –SalI digested) and (iii) the AatII- SacI fragment of the pBB-P_{lac}-RBS-lrp plasmid containing Cm-P_{lac}-RBS-lrp were cloned generating the pJQ-glmS-Cm-P_{lac}-RBS-lrp-ATPase. After transferring this construction by mating experiment in G2#25 as previously described (Givaudan and Lanois, 2000) using *E. coli* WM3064 as donor strain, the Cm-P_{lac}-RBS-lrp is transferred on G2#25 chromosome at the *glmS* site using allelic exchange and sucrose-resistant selection as previously described (Givaudan and Lanois, 2000). The obtained clones were then checked for GmS, SacR, CamR and by phenotypic tests. Sequence controls were performed on PCR using primers L-GlmS and R-ATPase and genomic DNA of the exconjugants as templates and they confirmed the insertion of the Cm-P_{lac}-RBS-lrp at the glmS site in the chromosome of the obtained clones. Phenotypic assays (haemolysin activity towards sheep blood, lecithinase activity, swimming motility, antibiotic production and dye binding assay) were performed as previously described (Givaudan et al., 1995; Thaler et al., 1998).

Primer name	Primer sequence	Use
L-PstI-RBS-lrp	cgctgcaGGGAAAATGTTATGGGTGTAGG	cloning of *lrp* gene from *X. nematophila*
R-BamHI-lrp	cgggatccTTAACGAGTCTTAATCACCAGACG	F1 wild type with its own RBS
L-GlmS-SpeI	GGACTAGTCCTTCACGACCCAGCTAACA	cloning of *glmS* site from *X. nematophila* F1 wild type
R-GlmS-AatII	GGCGACGTCAACCTTATTCCACCGTCACCG	
L-ATPase-SacI	GGCGAGCTCCAATGTGATTGATTGATTTTTAATC	cloning of *ATPase* site from *X. nematophila* F1 wild type
R-ATPase-SalI	GCAGTCGACAATGAAACGCCCTGATGTTC	
L-GlmS	ACGTTGTGGGTTCCTCTCTG	checking chromosomal insertion at *glmS* site
R-ATPase	TTTCTTGTTCAGACAAGGGTTG	

Table S3-1: Primers used in this study

2 Results

As shown in Table S2-2, the insertion of the Cm-P$_{lac}$-RBS-lrp at the *glmS* site in the chromosome of G2#25 strain belonging to the group 2 allowed a full restoration of all phenotypes (dye binding, motility, lecithinase et haemolysis activities) to achieve phenotypic pattern similar to the group 1 variant represented by G1#23.

3 References

Kovach ME, Phillips RW, Elzer PH, Roop II R.M, Peterson KM (1994). pBBR1MCS: a broad-host-range cloning vector. BioTechniques 16: 800-802

Givaudan A, Lanois A (2000). flhDC, the flagellar master operon of *Xenorhabdus nematophilus*: requirement for motility, lipolysis, extracellular hemolysis, and full virulence in insects. J Bacteriol 182: 107–115

Givaudan A, Baghdiguian S, Lanois A, Boemare N (1995). Swarming and swimming changes concomitant with phase variation in *Xenorhabdus nematophilus*. Appl Environ Microbiol 61:1408-1413

Thaler JO, Duvic B, Givaudan A, Boemare N (1998). Isolation and entomotoxic properties of the *Xenorhabdus nematophilus* F1 lecithinase. Appl Environ Microbiol 64: 2367–2373

Strains[a]	Dye binding [b]	Motility [c]	Antibiotic production [d]	Lecithinase [e]	Haemolysis activity [f]
G1#23	Blue	60	55	++	T
G2#25	Red	18	35	-	w
G2#25 glmS::P_{lac}-RBS-lrp	Blue	50	40	++	T

Table S3-2: Phenotypic assays in variants and lrp-complemented variants.

[a] All strains were cultured for 2 days at 28°Cbefore assays were interpreted unless indicated otherwise. Experiments were done 3 times, only one is shown

[b] Bromothymol blue

[c] Motility in swim agar (LB medium, 0.35% agar) plates; numbers are halo sizes (mm)

[d] Zone sizes (mm) of growth inhibition of *Micrococcus luteus*

[e] Activity measured as the production of a white halo of precipitation on plate with medium containing 0.01% lecithin; ++, halo up to 10 mm in diameter; - no precipitation observed

[f] T, total haemolysis; W weak haemolysis

Prolonged culture and long lasting infections select for poorly transmitted bacterial variants — Supplementary materials S4

November 30, 2018

In vitro kinetics of variants emergence

This experiment aimed distinguishing the emergence of group 2 from that of group 3 variants in prolonged static cultures of group 1 variants, using bacterial motility as a diagonostic character. For this purpose, we performed 25 independent cultures of G1#23, and we streaked these cultures every 24 hours onto NBTA plates. Each time red colonies were observed on the NBTA plate, a maximum of five red colonies and an identical number of blue colonies were sampled and their heamolytic capacity and their motility were measured. Petri dishes where swarming was observed were excluded from analysis, as they did not allow reliable motility measurement.

In this experiment, NBTA plates being incubated for 48 hours, we knew that red variants were present in a culture only two days after they have been sampled. Because of this delay, we stopped sampling bacteria from a culture only two days after red variants have appeared. In the results we present here, we include measurement

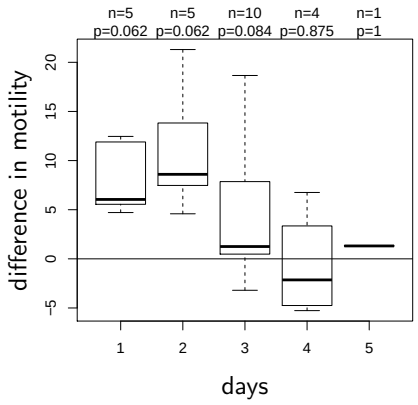


Figure S4-1: Difference in motility between red and blue isolates, as a function of the day they were sampled in a LB culture.

performed on bacteria sampled one day after red variants have been first detected in a culture. This increases sample size and provides us with better estimates of bacterial traits. But, as we want to focus on variants that just appeared, we will not consider bacteria sampled more than one day after red variants are first detected in the *X. nematophila* culture.

All cultures did contain red variants after five days of incubation. Seventy-five out of the 146 red colonies we sampled were non or weakly haemolytic, while all of the 143 blue colonies we sampled were fully haemolytic. In addition, we found that the difference in motility between red and blue colonies was significantly positive over the first two days of the experiment (Wilcoxon signed rank test: $p = 0.001953$) but did not significantly depart from zero afterwards (Wilcoxon signed rank test: $p = 0.625$, see figure above. As a result, we found that the difference in motility between red and blue colonies significantly decreased over time (Spearman rank correlation: $\rho = -0.614$, $p = 0.001095$).

Supplementary material for Chapter 2: "Changes in rearing conditions rapidly modify gut microbiota structure in *Tenebrio molitor* larvae"

All supplementary material and datasets mentioned in the text can be downloaded here: `https://dl.univ-tlse3.fr/filez/n3xabn`

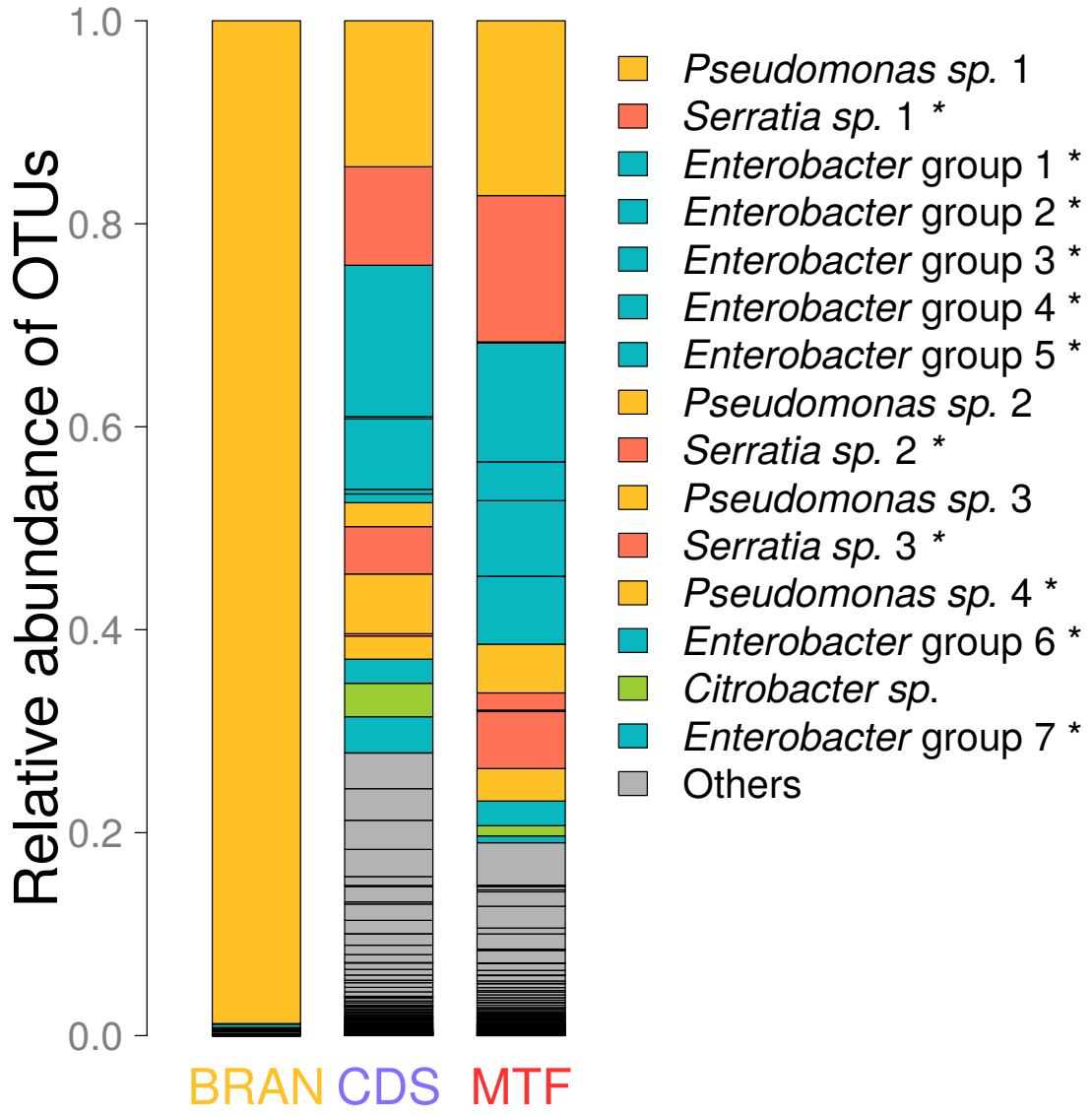


Figure S1 – **Relative abundance and taxonomic assignment of OTUs according to the gyrB gene** Insects from the various treatments were pooled for these bar plots: 5 insects for BRAN, 24 insects for CDS and 22 insects for MTF. The relative abundance of OTUs was calculated from the total number of reads for each insect pool. We show here taxonomic assignments to genus level or to the lowest taxonomic level for which the bootstrap score was ¿ 80 %. Some OTUs differ in sequence but were assigned to the same taxonomic group. These sequences are differentiated by a number. On each graph, the 15 OTUs with the largest relative abundances are shown in color and the others are grouped together in the "Others" category. OTU names followed by a star (*) belong to the Enterobacteriaceae family.

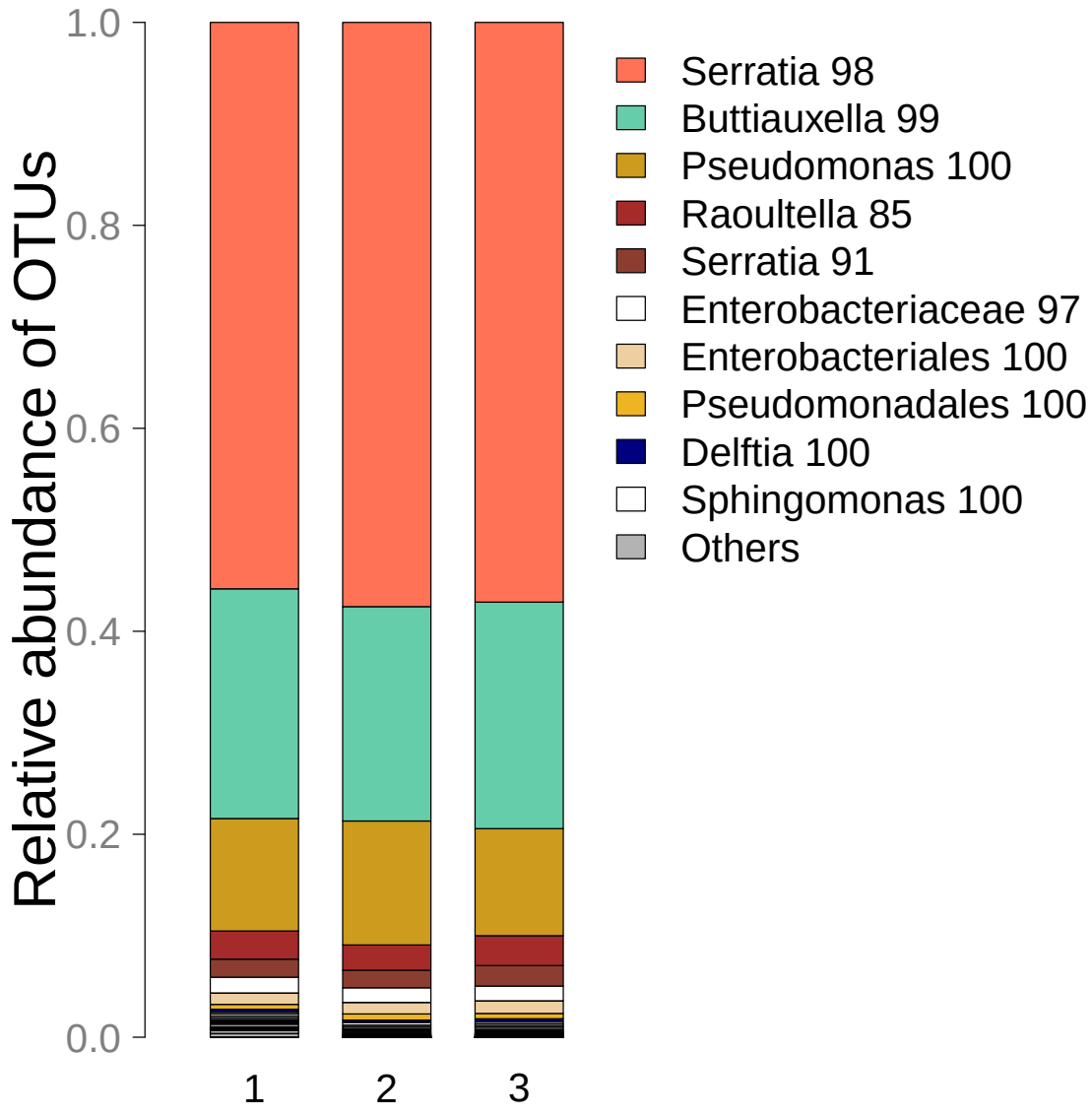


Figure S2 – **Example of a microbiota pattern in PCR replicates**. We checked the reproducibility of PCR, by performing three technical PCR replicates (the three bars of the chart) on a sample chosen at random, with the whole metabarcoding procedure performed separately for each replicate. We show here the results for the CDS1D3 sample.

Data-processing 16S V3V4 region on *T. molitor* microbiota with OBITools

Marine Cambon

27 janvier 2017

Assesing read quality and read pairing

Reads quality

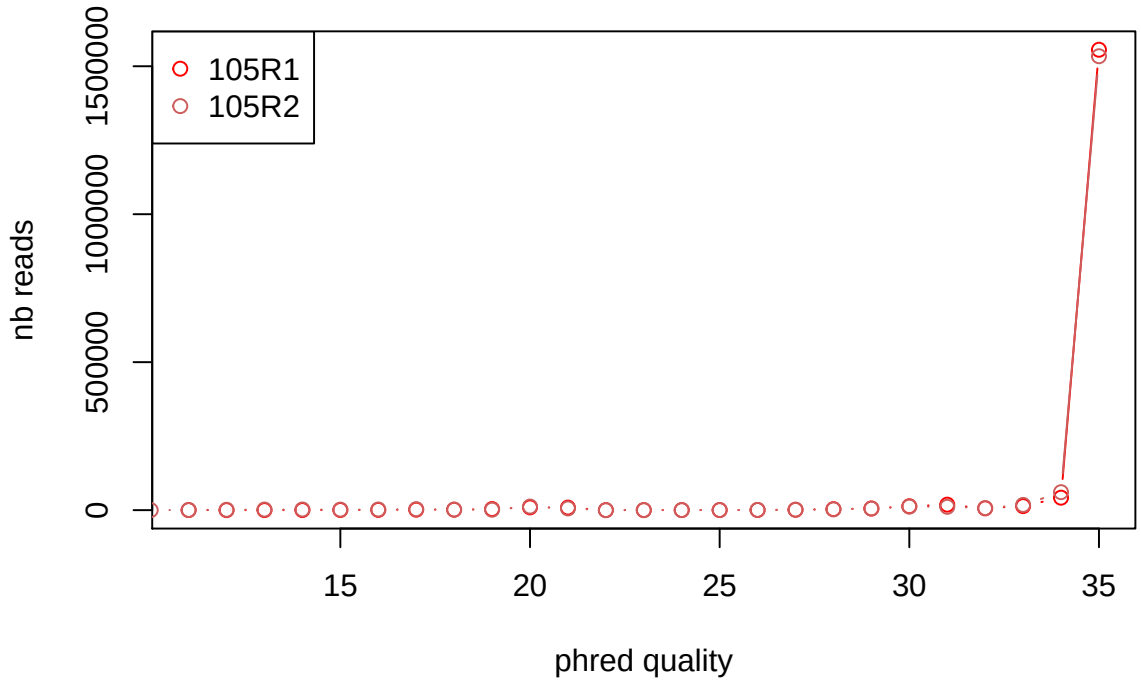
Calculation of mean phred scores on the first 10 (105), the last 10 (103), and all the bases (All) on R1 and R2 fragments before assemblage.

```
cat phredscore.csh
```

```
## #!/bin/tcsh
## #$-M cambonmarine@gmail.com
## #$-m a
##
##
## set a = $1
## set b = `echo $a | sed 's/R1/R2/'`
## set c = `echo $a | sed 's/R1/R1R2/'`
## set d = `echo $a | awk '{split($0,a,"_"); print a[1]}'`
##
##     #Add the mean phred score for each sequence, calculated on the first 10 bases (105),
##     #the last 10 bases (103) or all bases (all) :
## obiannotate --without-progress-bar --sanger \
## -S 'qphred105R1:-int(math.log10(sum(sequence.quality[0:10])/10)*10)' \
## -S 'qphred103R1:-int(math.log10(sum(sequence.quality[-10:])/10)*10)' \
## -S 'qphredAllR1:-int(math.log10(sum(sequence.quality)/250)*10)' $a | \
## obiannotate --set-id="'V3V4_Tenebrio_$d:r_%03d' % counter" > $d.tmpR1
##
## obiannotate --without-progress-bar --sanger \
## -S 'qphred105R2:-int(math.log10(sum(sequence.quality[0:10])/10)*10)' \
## -S 'qphred103R2:-int(math.log10(sum(sequence.quality[-10:])/10)*10)' \
## -S 'qphredAllR2:-int(math.log10(sum(sequence.quality)/250)*10)' $b | \
## obiannotate --set-id="'V3V4_Tenebrio_$d:r_%03d' % counter" > $d.tmpR2
##
##     #Compute the number of sequences for each score in each cathegory
##     #for visualisation in R :
##
## obistat --without-progress-bar -c qphred105R1 $d.tmpR1 > $c:r.qphred105R1
## obistat --without-progress-bar -c qphred103R1 $d.tmpR1 > $c:r.qphred103R1
## obistat --without-progress-bar -c qphredAllR1 $d.tmpR1 > $c:r.qphredAllR1
## obistat --without-progress-bar -c qphred105R2 $d.tmpR2 > $c:r.qphred105R2
## obistat --without-progress-bar -c qphred103R2 $d.tmpR2 > $c:r.qphred103R2
## obistat --without-progress-bar -c qphredAllR2 $d.tmpR2 > $c:r.qphredAllR2
```

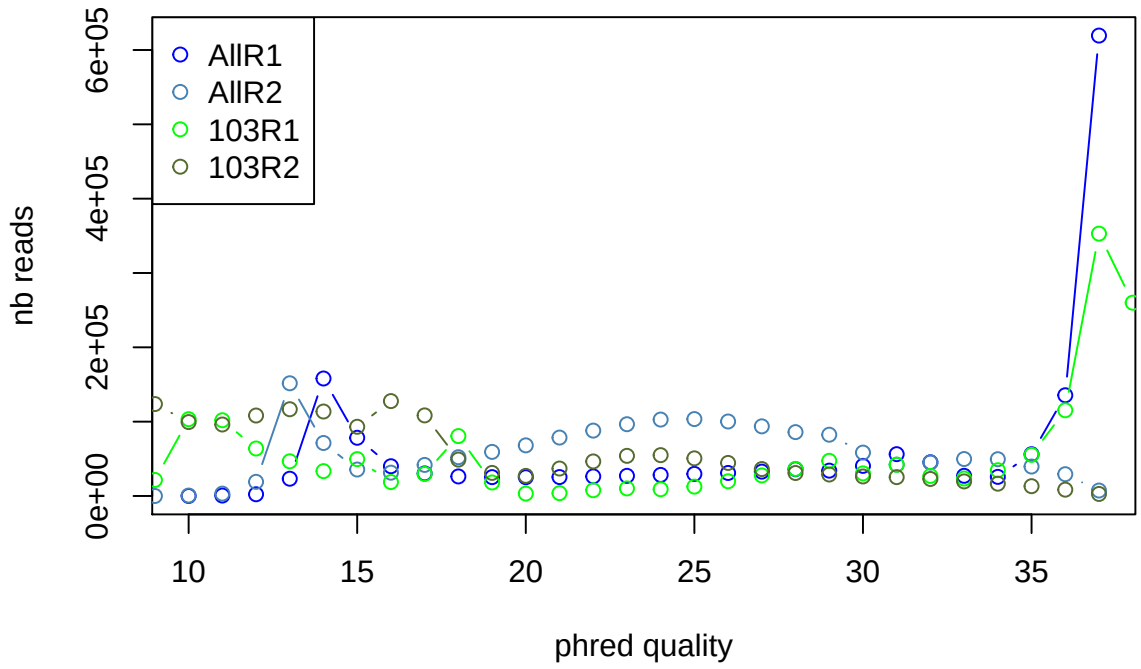
```
res_103R1 <- res_103R2 <- res_105R1<- res_105R2 <- res_AllR1 <- res_AllR2 <- NULL
res_tot <- list()
```

```
for (i in c("103R1", "103R2", "105R1", "105R2", "AllR1", "AllR2")) {
  res <- NULL
  fichiers <- list.files(path="phredscores/",
                         pattern=paste(".*qphred", i, sep=""),
                         full.names = T)
  for (f in fichiers) {
    dtmp <- read.table(f, h=T)
    res <- rbind(res, dtmp)
    res_tot[[i]] <- res
  }
}

#pdf("qphred_105.pdf", 5,5)
z <- unlist(with(res_tot[["105R1"]], tapply(total, qphred105R1, sum)))
plot(z~as.numeric(names(z)), type="b",
     xlab="phred quality",
     ylab="nb reads", col="red")
z <- unlist(with(res_tot[["105R2"]], tapply(total, qphred105R2, sum)))
points(z~as.numeric(names(z)), type="b", col="indianred")
legend("topleft", col=c("red", "indianred"),
       pch=c(1,1), c("105R1", "105R2"))
```




```
#dev.off()

#pdf("qphred_103_All.pdf", 5,5)
z <- unlist(with(res_tot[["AllR1"]], tapply(total, qphredAllR1, sum)))
plot(z~as.numeric(names(z)), type="b", col="blue",
     xlab="phred quality", ylab="nb reads")
z <- unlist(with(res_tot[["AllR2"]], tapply(total, qphredAllR2, sum)))
points(z~as.numeric(names(z)), type="b", col="steelblue")
z <- unlist(with(res_tot[["103R1"]], tapply(total, qphred103R1, sum)))
```

```
points(z~as.numeric(names(z)), type="b", col="green")
z <- unlist(with(res_tot[["103R2"]], tapply(total, qphred103R2, sum)))
points(z~as.numeric(names(z)), type="b", col="darkolivegreen")
legend("topleft", col=c("blue", "steelblue","green","darkolivegreen"),
       pch=c(1,1), c("AllR1", "AllR2", "103R1", "103R2"))
```




```
#dev.off()
```

Reads pairing

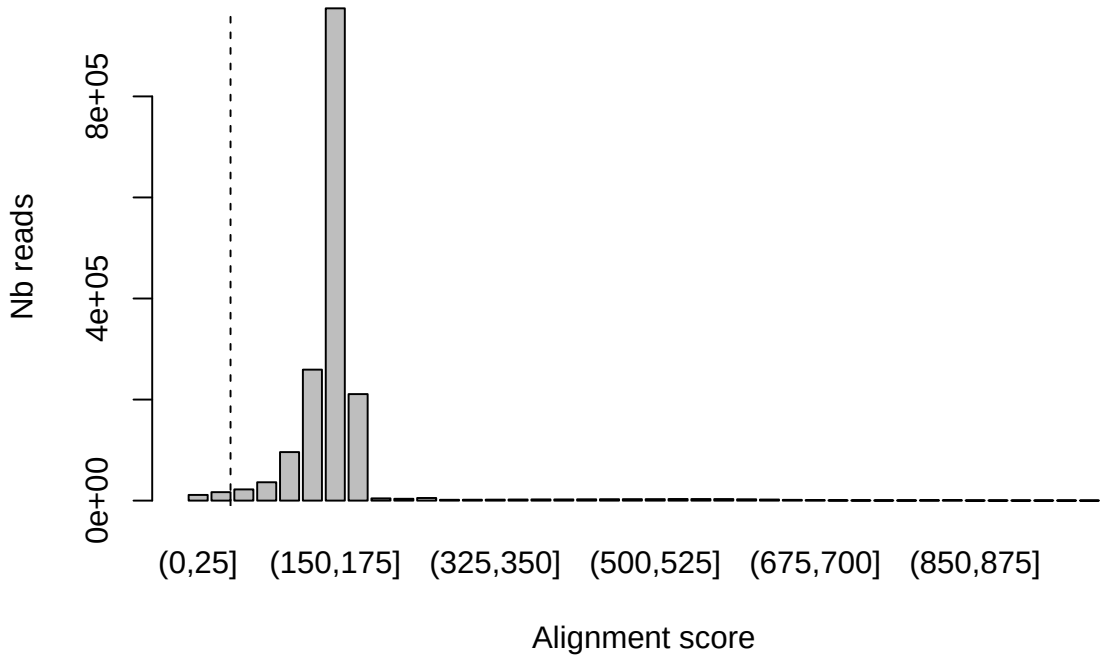
```
cat readpairing.csh
```

```
## #!/bin/tcsh
## #$-M cambonmarine@gmail.com
## #$-m a
##
## set d = $1
##
## set e = `echo $d | sed 's/R1/R2/'`
## set f = `echo $d | awk '{split($0,a,"\\."); print a[1]}'`
##
## illuminapairedend --without-progress-bar --fasta-output -r $e $d \
## | obiannotate --length > $f:r_partR1R2.fasta
## obitab -d -o -n NA $f:r_partR1R2.fasta
```

Scores d'alignement

```
d <- read.table("align_scores", h=T)
d$class <- cut(d$score,seq(0,1000,by=25))
```

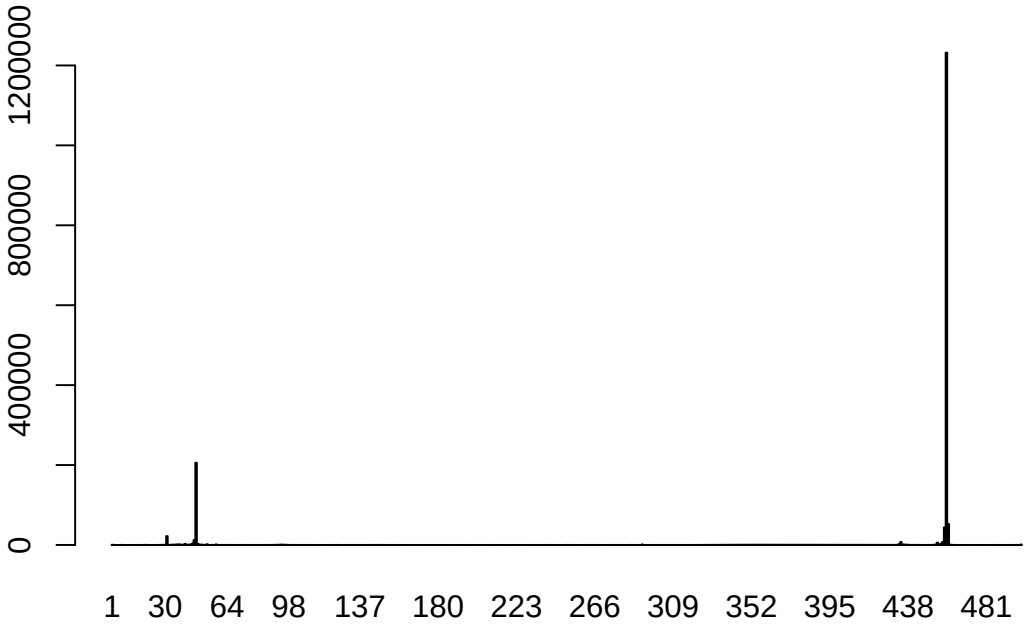
```
barplot(tapply(as.numeric(d$count), d$class, sum),
        xlab="Alignment score",
        ylab="Nb reads")
abline(v = 2.4, lty=2)
```



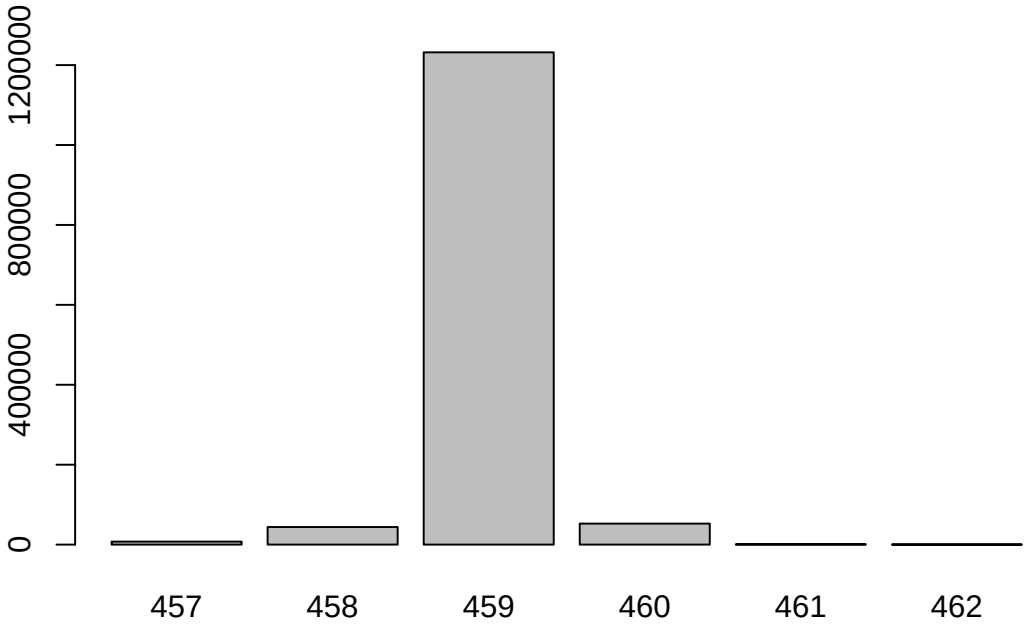
Sequence length after reads pairing

Length verification of the reads after assemblage to check if the coverage between R1 and R2 fragment is sufficient: Most of reads are around 459 pb. Therefore there is around 40 bases of coverage which is enough.

```
d <- read.table("seq_length_avant_nettoyage", h=T)

#pdf("seq_length_tot.pdf", 5, 5)
barplot(d$count, names.arg=d$seq_length)
```




```
#dev.off()

#pdf("seq_length_456_462.pdf", 5, 5)
with(subset(d, seq_length > 456 & seq_length< 463), barplot(count, names.arg=seq_length))
```

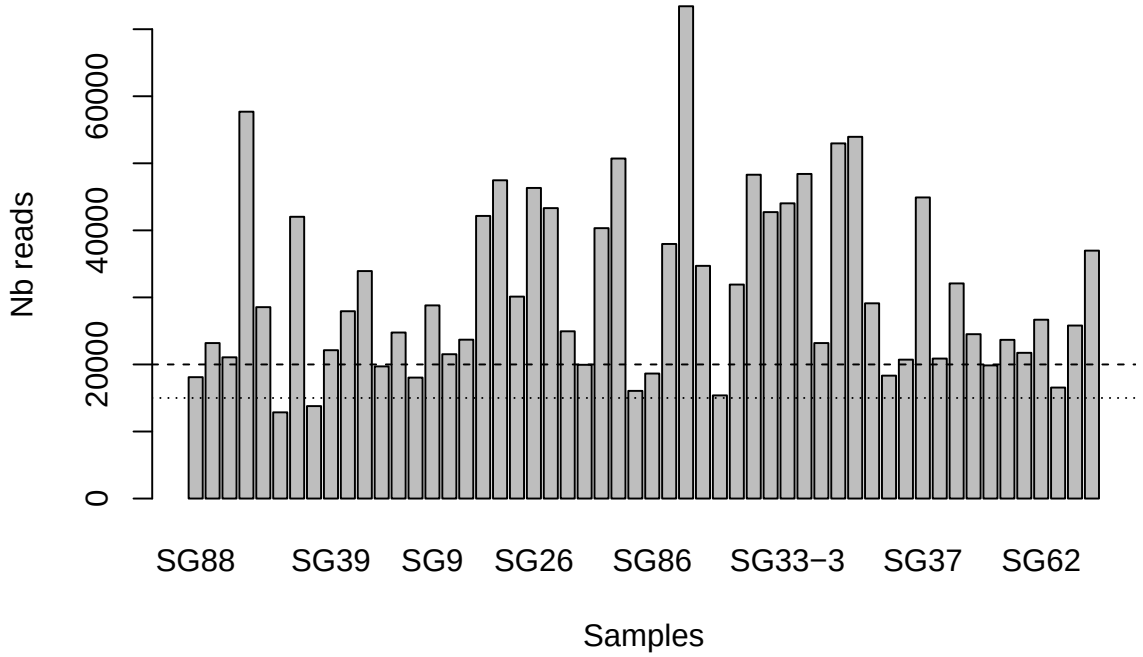



```
#dev.off()
```

Number of reads per sample

```
d <- read.table("reads_per_samples_before_cleaning", h=T)
barplot(d$count, names.arg = d$sample,
        xlab="Samples", ylab = "Nb reads")
```

```
abline(h=20000, lty=2)
abline(h=15000, lty=3)
```



Primers cleaning

The ngsfilter tool of OBITools is made for demultiplexing samples. I here add a fake tag combination to each sequence and construct the ngsfilt file used to trim primers.

Rq: I figured out later that ngsfilter can be used with an empty column of tags if samples are not multiplexed.

```
cat add_tag.csh
```

```
## #!/bin/tcsh
## #-M cambonmarine@gmail.com
## #-m a
##
## set a = $1
##
## sed 's/^\([actg][actg][actg][actg]*\)$/acacacac\1tgcgacta/g' $a > $a:r_tag.fasta
```

```
cat clean_primers.csh
```

```
## #!/bin/tcsh
## #-M cambonmarine@gmail.com
## #-m a
##
## set a = $1
## set b = `echo $a | sed 's/_partR1R2_tmp_tag.fasta//'`
##
##     #create a ngsfilt with tags for each sample
##     echo 'pilote_tenebrio_V3V4   '$b\
##      '   acacacac:tagtcgca   ACGGRAGGCAGCAG  TACCAGGGTATCTAATCCT F   @' \
```

```
##             > $b:r_ngsfilter.txt
##
## # trim of primers and annotation of sequences with samples name
##     ngsfilter -t $b:r_ngsfilter.txt -u $a:r_ngsfilt_nomatch.fasta -e 2 --nuc $a > \
##     $a:r_ngsfilt.fasta
##     obistat -c error $a:r_ngsfilt_nomatch.fasta | awk -v FILE=$a '{if (NR!=1) {print FILE $0}}' > \
##     $a:r_nomatchstat.txt
```

Sequences cleaning

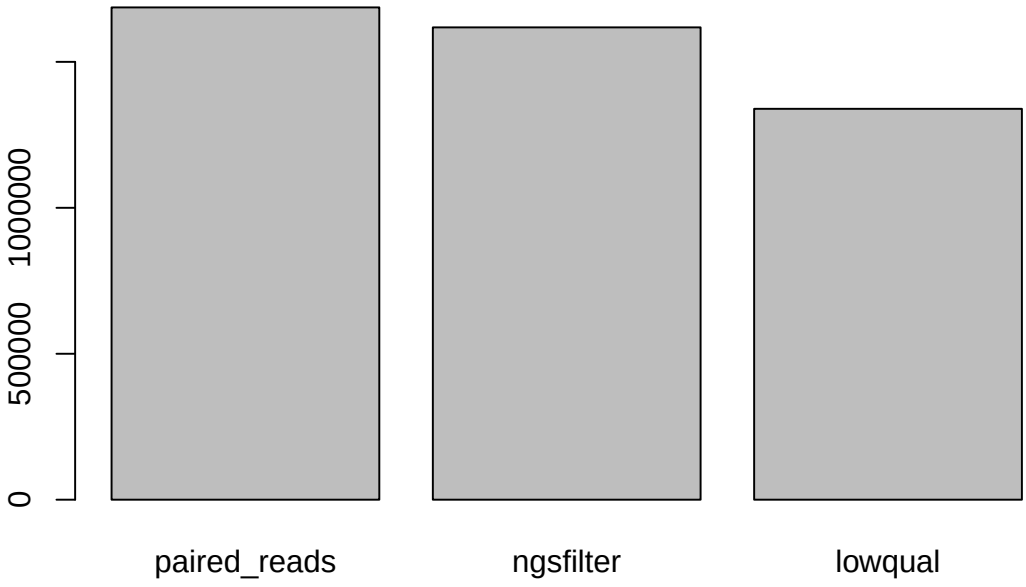
Removing low quality reads

Low quality reads have an alignment score < 50 and/or have some unknown bases and/have have the wrong size (shorter than 400 pb or longueur than 470 pb)

```
cat lowqual.csh
```

```
## #!/bin/tcsh
## #$-M cambonmarine@gmail.com
## #$-m a
##
## set a = $1
## obigrep -s '^[acgt]+$' -l 400 -L 470 -p 'score>=50' $a > $a:r_n50ali.fasta
##
## echo "\nNombre de séquence après suppression des read de mauvaise qualité" >> stat_file.txt
## echo "-----------------------------------------------------------------" >> stat_file.txt
##
## obicount $a:r_n50ali.fasta >> stat_file.txt
```

```
d <- read.table("nb_read_par_etape")
barplot(d$V1, names.arg = d$V3)
```

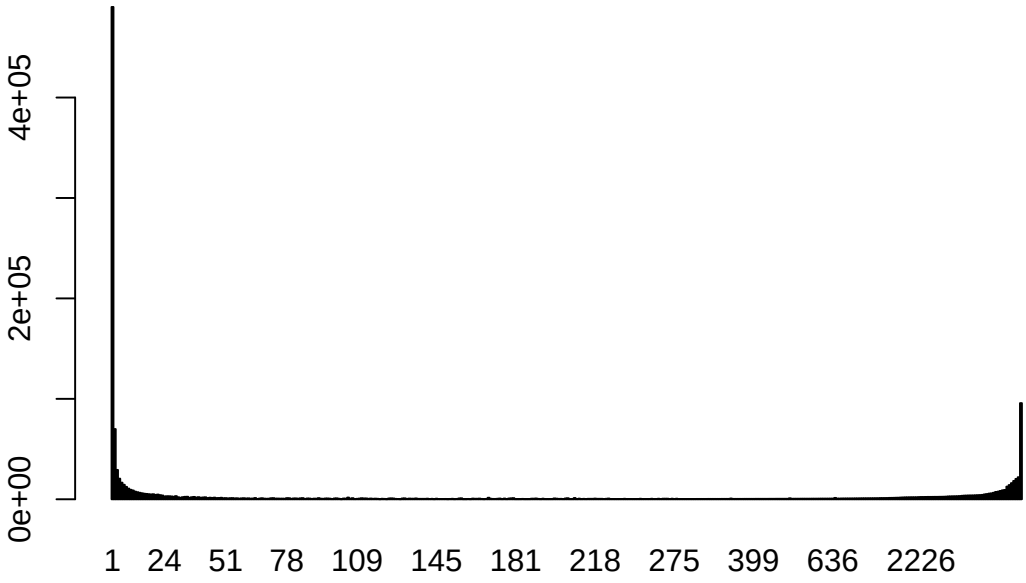


Reads dereplication and removing singletons

```
cat derepliq.csh
```

```
## #!/bin/tcsh
## #$-M cambonmarine@gmail.com
## #$-m a
## #
##
## set a = $1
##
## obiuniq -m sample $a > $a:r_uniq.fasta
##
##
## echo -e '\nNombre de séquences après déréplication' >> stat_file.txt
## echo -e '----------------------------------------' >> stat_file.txt
## obicount $a:r_uniq.fasta >> stat_file.txt
##
## echo -e '\nNombre de séquences pour les 10 counts les plus faibles :' >> stat_file.txt
## echo -e '------------------------------------------------------------' >> stat_file.txt
## obistat -c count $a:r_uniq.fasta | sort -nk1 | head -n 10 >> stat_file.txt
```

```
d <- read.table("resume_nb_count_uniq", h=T)
d <- d[order(d$count),]
barplot(d$total, names.arg = d$count)
```



A lot of reads are only found once in the dataset (= singleton) and probably correspond to a sequencing mistake. We remove all the singletons before clustering.

```
cat noSingleton.csh
```

```
## #!/bin/tcsh
## #$-M cambonmarine@gmail.com
## #$-m a
## #
##
```

```
## set a = $1
##
## obigrep -p 'count>1' $a > $a:r_noS.fasta
##
## echo -e '\n Nombre de séquences restantes après suppression des singletons :' >> stat_file.txt
## echo -e '-----------------------------------------------------------------------' >> stat_file.txt
## obicount $a:r_noS.fasta >> stat_file.txt
```

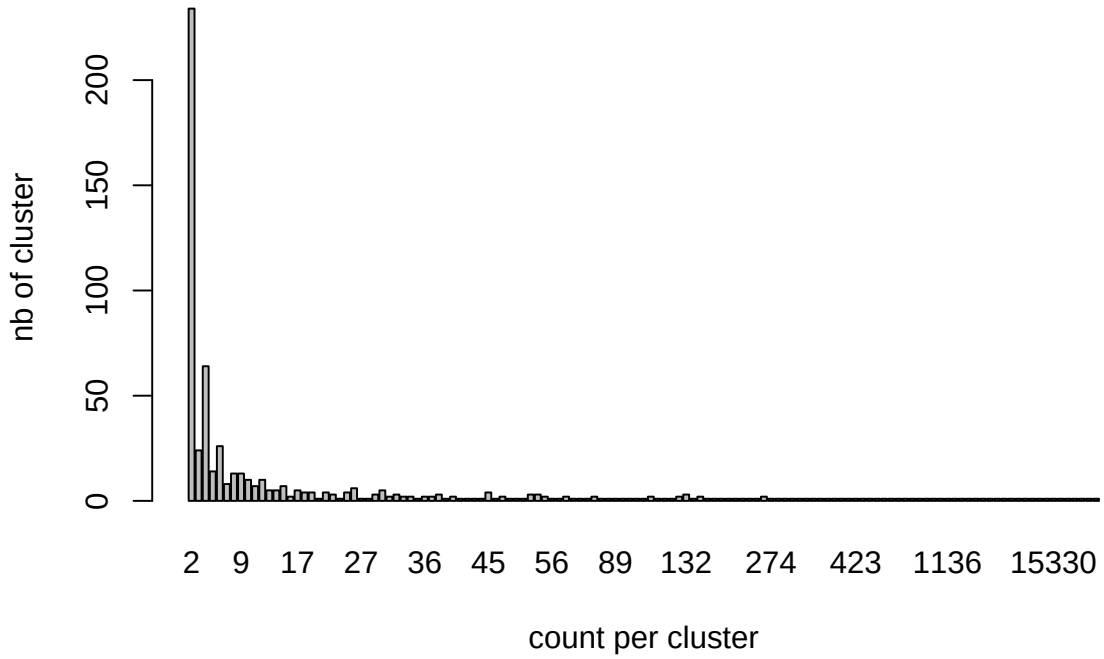
Sequences clustering Sumaclust

97 % similarity threshold

```
cat clustersumatra_97.csh
```

```
## #!/bin/tcsh
## #$-M cambonmarine@gmail.com
## #$-m a
## #$-pe parallel_rr 4
## #$-cwd
##
## set a = $1
##
## /usr/local/bioinfo/src/sumatra/current/sumaclust/sumaclust -t 0.97 -p $NSLOTS $a > $a:r_cl97.fasta
##
## echo '\nNombre de clusters après Sumaclust 97% :' >> stat_file.txt
## echo '----------------------------------------' >> stat_file.txt
## obistat -c cluster $a:r_cl97.fasta | wc -l >> stat_file.txt
```

```
d <- read.table("count_par_cluster", h=T)
d <- d[order(d$total),]
barplot(table(d$total), xlab="count per cluster", ylab = "nb of cluster")
```



Data formating for sequences analyses and assignation

Table aggregation to have at the end only one sequence per cluster, and fasta file converting to tab format *pool_tmp_tag_ngsfilt_n50ali_uniq_noS_cl97_agg.fasta*

```
cat agg_tab.csh
```

```
## #!/bin/tcsh
##
## set a = $1
##
## obiselect -c cluster -n 1 --merge sample -M -f count $a > $a:r_agg.fasta
## obitab -d -o -n NA $a:r_agg.fasta > $a:r_agg.tab
```

Suppressing annotations on sequences in order to use the regular fasta file on RDPclassifier (sequence taxonomic assignation)

```
obiannotate -C pool_tmp_tag_ngsfilt_n50ali_uniq_noS_cl97_agg.fasta >
pool_tmp_tag_ngsfilt_n50ali_uniq_noS_cl97_agg_noAnn.fasta
```

Assignation

Performed on RDPclassifier with RDPII reference database, using the online tool of RDP

Heterogeneity within infections: the case of *Xenorhabdus nematophila*, a vector-borne insect pathogen

Numerous studies have considered infections as pairwise interactions between a single pathogen and its host, sometimes leading to an incomplete picture of infectious processes. In this work, we focused on more complex types of interactions that arise because infections are usually heterogeneous. More precisely, we have investigated two main issues: (i) **how pathogen transmission is impacted by phenotypic heterogeneity which arises within the pathogen population during the infection**, and (ii) **how do pathogens interact with the bacterial community which is naturally associated to the host before infection?** To assess these questions, we have been studying *Xenorhabdus nematophila*, an insect-killing bacterial pathogen which is transmitted by a nematode vector, *Steinernema carpocapsae*.

One interesting feature of *X. nematophila* is that it produces different sub-populations during the course of an infection, each one having distinctive phenotypic features (e.g. one form produces antibiotics and is mobile, while the other does not produce antibiotics nor flagella). In this work, we first tried to identify the molecular mechanisms responsible for this diversification of phenotypes, and tested if phenotypic heterogeneity in *X. nematophila* has some adaptive value. We showed that some of these phenotypic forms were mutants, which seem to be under strong positive selection during infection. We also showed, however, that these mutants impair nematodes reproduction, which in turn reduces transmission. Therefore, the dynamics of phenotypic heterogeneity in *X. nematophila* seems to be determined by contradictory short-term and long-term selective pressures.

A second interesting feature of *X. nematophila* is that it produces a lot of antimicrobial compounds which should allow it to dominate the bacterial community inside the insect it has killed. This can be key to ensure the re-association of *X. nematophila* with its nematode vector inside the insect cadaver. We investigated the bacterial composition of the microbial communities present in insects cadavers after infection by *X. nematophila*. We found that despite the numerous antibiotics it is able to secrete, *X. nematophila* is far from dominating microbial community after host death. It rather cohabits with microorganisms from the microbiota of both the insect host and the nematode vector. This raises numerous questions about the impact of these other microorganisms on *Xenorhabdus-Steinernema* interactions, and therefore on their potential influence on how this mutualistic association has evolved.

Auteur : Marine Cambon
Titre : Hétérogénéité au sein d'une infection : le cas de *Xenorhabdus nematophila*, un pathogène d'insecte à transmission vectorielle
Directeurs de thèse : Jean-Baptiste Ferdy et Sophie Gaudriault
Lieu et date de la soutenance : Université Paul Sabatier, le 14 décembre 2018

De nombreuses études ont jusqu'ici considéré les infections comme étant des interactions deux-à-deux, entre un hôte et un pathogène, minimisant ainsi la complexité du processus infectieux. En effet, les infections sont souvent très hétérogènes, menant à des interactions plus complexes. Au cours de ce travail, nous cherchons à répondre à deux questions : (i) La transmission d'un pathogène peut-elle être impactée lorsque de l'hétérogénéité phénotypique apparaît dans sa population au cours de l'infection ? (ii) Comment les pathogènes interagissent-ils avec la communauté bactérienne généralement associée à l'hôte avant l'infection ? Pour étudier ces questions, nous nous sommes intéressés à *Xenorhabdus nematophila*, une bactérie pathogène d'insectes transmise par un vecteur, le némaotde *Steinernema carpocapsae*.
Au cours d'une infection par *X. nematophila*, différentes sous-populations ayant différentes caractéristiques phénotypiques sont produites. Nous avons cherché à déterminer les mécanismes moléculaires responsables de cette diversification phénotypique, ainsi que sa potentielle valeur adaptative pour *X. nematophila*. Nous avons montré que certaines de ces formes phénotypique sont des mutants qui semblent être sous forte sélection positive au cours de l'infection. À l'inverse, ces mutants ont un impact négatif sur la reproduction du vecteur nématode, ce qui réduit leur transmission. La dynamique d'hétérogénéité phénotypique chez *X. nematophila* semble donc déterminée par des pressions de sélections contraires à court terme et à long terme.
La production de molécules anti-microbiennes chez *X. nematophila* devraient lui permettre de dominer la communauté bactérienne à l'intérieur de l'insecte et faciliter sa ré-association avec son vecteur. Nous avons donc décrit la composition de la communauté microbienne présente dans des insectes morts d'une infection par *X. nematophila*, et montré qu'en dépit de sa production d'antibiotiques, *X. nematophila* est loin de dominer la communauté microbienne après la mort de l'insecte. Elle cohabite avec des bactéries provenant à la fois du microbiote de l'hôte insecte, et de celui du vecteur nématode. Cela soulève de nombreuses questions sur le rôle d'autres microorganismes dans les interactions *Xenorhabdus-Steinernema*, et sur leur influence dans l'évolution de cette symbiose mututaliste.

Mots-clés : *Xenorhabdus*, microbiote, hétérogénéité phénotypique, pathogène

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Intitulé et adresse de l'U.F.R ou du laboratoire :
Laboratoire Évolution et Diversité Biologique (EDB)
UMR 5174 CNRS-IRD-UPS
Université Paul Sabatier, Bâtiment 4R1
118 route de Narbonne, 31062 Toulouse CEDEX 9 - France